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FOREWORD

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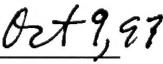
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Introduction

The rate limiting step in control of somatic cell duplication is the commitment to enter S-phase. This step occurs in the G₀ or G₁ stage of the cell cycle depending upon the growth state of the cell in question. The process of S-phase entry is complex but, in fibroblasts, can be divided into four stages as defined by Pardee (1). These stages include exit from quiescence (dependent upon growth factors such as PDGF), entry into G₁ (dependent upon e.g. EGF), progression through mid and late G₁ to the restriction point (dependent upon IGF-I and protein synthesis), and assembly of factors required for S phase. The restriction point is thought to reflect a point in the cell cycle where further transitions are not dependent upon mitogenic stimuli, and has therefore received the greatest attention. Initiation of DNA replication begins 1-3 hours after passage through the restriction point, and it is thought that these two events are differentially controlled. Data from a number of systems indicate that both of these transitions are dependent upon the activities of members of the cyclin dependent kinase (Cdk) family of proteins. Although much is known about the proteins that regulate the G₁/S transition, the precise biochemical mechanisms which underlie this transition are not fully understood.

Cyclin-dependent kinases and positive cell cycle control. Progression through the cell cycle is dependent upon the assembly and activation of a family of Cdks (reviewed in 2-3). To date, four Cdks (Cdk2, 3, 4 and 6) have been implicated in regulation of the G₁/S transition in mammalian cells. These kinases are inactive in their monomeric form and require association with members of the cyclin family of proteins, as well as phosphorylation on a conserved threonine residue, for activation. This activating modification can be catalyzed by Cdk7/cyclin H, a Cdk activating kinase (CAK) (4). In cells released from G₀, D and E type cyclins are sequentially expressed during G₁ (reviewed in 2). Cyclin E is expressed transiently and is degraded early in S-phase (5,6). In some cell types, cyclin D levels remain high throughout the remainder of the cell cycle (7). D-type cyclins (D1, D2, and D3) are thought to primarily activate Cdk4 and Cdk6, while cyclin E activates Cdk2 and perhaps Cdk3 (see below). Cyclin A is synthesized during S and G₂ and activates Cdk2 and Cdc2. Consistent with a positive role for D-type cyclins in cell cycle entry, these genes are growth factor inducible in certain cell types (reviewed in 2).

A number of laboratories have addressed the issue of whether cyclins are required for early cell cycle events. Injection of antibodies against cyclin E can block entry of normal diploid fibroblasts into S-phase (8,9). Similar experiments with cyclin A indicate that it functions in coordinating at least two transitions, including DNA replication and passage through G₂ (9-11). In cells engineered to overexpress D or E-type cyclins, entry into S-phase is accelerated by ~2 h compared with control cells, providing additional evidence that G₁ Cdks are rate limiting for certain steps in the G₁/S transition (12-14).

Negative cell cycle control pathways. Much of our knowledge about negative regulation of cell cycle entry emanates from studies of two tumor suppressor genes, Rb and p53 (15-17). Mutations in these are found frequently in diverse types of human cancers, and reintroduction of wild-type genes into p53^{-/-} or Rb^{-/-} tumor cells can suppress the neoplastic phenotype suggesting that loss of function of these genes contributes to tumorigenesis (18-21).

Mutations in p53 are the most common lesions observed in human malignancies, occurring in greater than 50% of all tumors (17) including those of the breast. The percentage is much higher if loss of p53 function via association with viral oncoproteins (E1B of adenovirus and E6 of papilloma virus) or amplification of the p53 binding protein MDM2 are included. p53 deficient mice are prone to the spontaneous development of a

variety of tumor types (22). Cellular responses to DNA damage such as apoptosis and the G1 checkpoint are dependent on p53. p53 also controls a spindle checkpoint and prevents genetic alterations such as gene amplification (23-28).

Rb functions to coordinate cell cycle transitions and transcriptional pathways that are required for cell cycle progression (15). The Rb protein displays two prominent features: 1) functional inactivation through various mechanisms contributes to deregulated proliferation and an inability of the cell to appropriately regulate G1 decisions, and 2) periodic alterations in its phosphorylation status that correlate with particular phases of the cell cycle. In early and mid G1, Rb is hypophosphorylated, as determined by mobility on SDS-PAGE, but as cells enter late G1 (approximately co-incident with the restriction point), Rb becomes progressively hyperphosphorylated and remains so until passage through mitosis. At mitosis, Rb is dephosphorylated, possibly due to the action of a type II protein phosphatase that has been shown to physically associate with Rb (15). In addition to Rb, there are two other prominent pocket proteins p107 and p130 which are thought to have roles in regulating G1 and S phases (15). These two proteins share some mechanistic similarities with Rb in that they are phosphoproteins and are targeted by tumor virus oncoproteins.

The finding that Rb phosphorylation correlates with cell cycle progression has led to a model whereby Rb serves a timing function, linking the cell cycle regulatory machinery with the transcriptional machinery responsible for coordinating expression of genes required for S-phase (15). This is thought to be accomplished through the interaction of Rb with particular target proteins and these interactions are thought to be disrupted temporally through Rb phosphorylation. The best understood Rb binding proteins are members of the E2F family of transcription factors that regulate expression of genes required for S-phase, although it is not clear whether E2F represents the target of Rb responsible for growth suppression (15). A large body of data implicate cyclin dependent kinases in Rb phosphorylation and inactivation of Rb's growth suppressive function. We have recently shown using a microinjection-based approach that phosphorylation of Rb by cyclin D/Cdk4 relieves Rb mediated growth suppression but cyclin E/Cdk2 and cyclin A/Cdk2 are inactive in this assay even though they phosphorylate Rb extensively on sites overlapping with those phosphorylated by Cdk4/cyclin D1 (30). We identified a single Cdk4/cyclin D1 phosphorylation site that is required for Rb inactivation in this assay.

Although G1 Cdks are likely mediators of Rb phosphorylation, it appears that other substrates are also rate limiting for DNA replication when cyclins are overexpressed. We have shown that a mutant of Rb lacking all 14 consensus Cdk phosphorylation sites is bypassed by co-expression of either cyclin E/Cdk2 or cyclin D1/Cdk4 in SAOS-2 cells (31). In this setting, high levels of cyclin/kinase activity is sufficient to activate DNA synthesis without Rb phosphorylation and activation of the E2F pathway (32).

Cyclin-dependent kinase inhibitors: mediators of negative cell cycle control. There is now clear evidence that cyclin/Cdk complexes can promote cell cycle transitions whether by Rb dependent or Rb-independent mechanisms. Acting in opposition to cyclin kinases are cyclin kinase inhibitors (CKIs). These proteins fall into two classes: the CIP/KIP class and the INK4 class (33-41). These versatile molecules have potential roles in cell cycle arrest, checkpoint function and development and are likely to cooperate with Rb, p53, and other negative regulators in maintaining the non-proliferative state throughout adult life.

At the time of submission of this grant in December 1993, we and others had just found the first mammalian Cdk inhibitors p21 (33-36) as either a Cdk binding protein or as a p53 regulated gene. At that time there were many unanswered questions regarding the role p21 in cell cycle control and cancer. What was known was that p21 could bind and inhibit a number of Cdks, that it was transcriptionally regulated by p53 overexpression, and that its

chromosomal location did not mark it as an obvious tumor suppressor. In addition, it was not known whether p53 is the only regulator of p21 expression, how p21 might be used during development, or whether loss of p21 expression contributes to cancer. Since that time, however, there has been an explosion in our understanding of the mechanisms of action and biological function of p21 (42, 43). In addition, we now know that p21 is only one member of a family of inhibitors that share structural and functional characteristics. p27KIP2 was identified as a protein associated with Cdk2/cyclin E or Cdk4/cyclin D (44, 45). Subsequently, we and others identified p57KIP2 as the third member of the CIP/KIP family in mammalian cells in a two-hybrid screen designed to identify cyclin D1 interacting proteins (46, 47). These inhibitors primarily inhibit Cdk2, Cdk4, and Cdk6 containing Cdk complexes. The second group of inhibitors, the INK4 family, are selective for Cdk4 and Cdk6. This group includes the tumor suppressor protein p16.

Our understanding of these molecules has moved forward in several ways (reviewed in 48). Knockout mice have now been created for all of the CIP/KIP and INK4 proteins and the phenotypes are emerging. In addition, we now have a good picture of how these proteins work biochemically and what proteins they interact with *in vivo*. Moreover, we know the structure of a cyclin A/Cdk2/p27 complex which allows an understanding of the relationships between structure and function for the CIP/KIP family. Finally, we are beginning to understand the transcriptional control pathways that dictate when and where these inhibitors are expressed.

As detailed in previous progress reports and summarized below, we have made excellent progress on the original five aims of this proposal, the major goal of which was to make and analyze p21 knockout mice. Much of our data has been summarized in great detail in the first two progress reports for this grant (1995 and 1996). With the exception of specific aims 4 and 5, essentially all of the major components of the specific aims have been completed and published (Table 1). Therefore, in this report, I will first briefly summarize our findings related to the specific aims indicated above with an emphasis on published findings and will focus the body of the report on unpublished data related to largely aims 4 and 5. We have included published papers in the appendix.

Body

Progress thus far (Aims 1-3, 5): Most of our studies thus far have focused on aims 1-3 and aim 5. The tasks laid out in aims 1-3 have been largely if not completely finished and were published in two papers (see Table 1). In short, for Aim 1, we found that p21 expression during development correlates with the process of terminal differentiation in a subset of tissues (49). Together with our analysis of p57 expression (46), we found that this class of CKIs is differentially expressed in a number of cell types *in vivo*. These studies also allowed us to develop a model whereby CKIs function directly in the process of differentiation (50), a property that we subsequently identified through the analysis of p57 deficient mice. For Aims 2 and 3, in collaboration with Dr. Phil Leder, we have generated p21 deficient mice and analyzed the phenotypes of both deficient animals and of cells derived from these animals (51). The results provide evidence that p21 is not required for development nor does loss of p21 predispose mice to transformation. Using mouse embryo fibroblast from these animals, however, we found that p21 is required for the G1 DNA damage checkpoint activated by p53. Other p53 dependent functions such as apoptosis are unaffected in these cells. For Aim 5, we have performed a number of studies (some of which are collaborative with other groups) that have characterized the interactions of p21 with Cdks (52), examined its biochemical function in the G1 DNA damage checkpoint (53, 54), and examined the effects of p21 on PCNA dependent DNA replication *in vitro* (55). p21 is associated with PCNA in cells, although the significance of this is

currently unknown. In addition, we have identified a p21 homolog - p57 - and characterized its interaction with Cdks (46).

Aim 4:
p21 expression in the cell cycle.

The major goal of Aim 4 was to examine p21 expression in mammary cells and to explore the question of whether p53 regulates p21 levels in these cells. We have addressed this question in part through an analysis of p21 expression in normal and tumor mammary epithelial cells. The data accumulated thus far indicates that p21 levels may be regulated in the epithelial cell cycle. It is unclear at present whether alterations in p21 contributes substantially to the development of cancer in this tissue (but see below).

Table 1: Summary of Our Published Data

Aim	Major Original Objectives	Publications
1	determine expression patterns of p21 during development and in adult; determine p53 dependence	<i>Science</i> 267, 1024-1027 (1995)
2	construct p21 deficient ES cells by homologous recombination; use cells to generate chimeric mouse and breed to homozygosity	<i>Cell</i> 82, 675-684 (1995)
3	characterize phenotype of p21 deficient mouse, including developmental effects, tumorigenesis, effects of p21 loss on DNA damage checkpoint control	<i>Cell</i> 82, 675-684 (1995)
4	Analysis of p21 expression in breast tumors and cell lines	see progress report
5	Identification of regulators of p21, analysis of targets of p21 including p21 associated proteins, regulation by phosphorylation, regulation of p21 expression by DNA damage, effects of loss of p57 on p21 levels	<i>Mol. Biol. Cell</i> 6, 387-400 (1995) <i>PNAS</i> 91, 8655-8659 (1994) <i>Cancer Res.</i> 54, 1169-1174 (1994) <i>Cell</i> , 76, 1013-1023 (1994) <i>Nature</i> , 387, 151-158 (1997) <i>Genes Dev</i> 9, 650-662 (1995) <i>Meth Enz.</i> 283, 230-244 (1997)
---	reviews emanating from this project	<i>Trends in Cell Biology</i> 6, 388-392 (1996) <i>Cancer Surveys</i> 29, 91-108 (1997) <i>Curr Opin Cell Biology</i> , 6, 847-852 (1994) <i>Curr Opin Gen Devel</i> 6, 56-64 (1996) <i>Science</i> 274, 1664-1672 (1996)

How the levels of p21 is regulated during cell cycle progression is not completely understood. It is possible that different cell types regulate p21 expression in different ways or that changes on the genetic background of cells either through transformation or immortalization can change how p21 levels are controlled. In this aim, we collaborated with Dr. Khandan Keyomarsi to examine how p21 levels are regulated in mammary epithelial cells. This cell type is normally the cell type that gives rise to breast cancer and there existed the possibility that p21 levels are altered in breast cancer and/or breast cancer cell lines. Initially, we examined p21 mRNA levels in synchronized normal human breast epithelial cells (Figure 1B). These cells were chosen because a high degree of synchrony can be achieved after serum deprivation and refeeding, and because they have been extensively characterized previously for cell cycle regulation of both cyclins and CDKs (56). In these cells, p21 mRNA levels were constant across the cell cycle. This expression pattern basically matched that of the protein but the levels of p21 protein in G₀ were lower than expected based on the mRNA level (Figure 1D). This suggests some level of translational or post-translational control in G₀ in this cell line. p21 regulation in mammary cells was examined further using two additional cell lines (76N and MCF 10A) and synchronization by both growth factor deprivation and double thymidine block. Growth factor deprived (G₀) MCF-10-A cells contain very low levels of p21 (Figure 1E). After stimulation, p21 expression is detectably induced at 20-24 h, corresponding to the time of entry into S-phase in these cells (see Figure 1E, legend). The timing of induction of cyclins A and D1 closely coincides with that of p21 in these cells (Figure 1E). Very similar results were obtained with growth factor deprived 76N cells (data not shown). Due to loss of synchrony, it is difficult to assess the status of p21 in mitosis in the stimulated cells. Therefore, p21 levels were also analyzed in 76N cells synchronized by double thymidine block (Figure 1E). p21 levels were near maximal immediately after release (in S-phase), but at 6-9 h after release, p21 levels decrease sharply. This time corresponds to mitosis based on the G₂ DNA content determined by flow cytometry (data not shown) and the expression pattern of cyclin A (Figure 1E). As cells re-enter G₁, p21 rapidly accumulates to maximal levels. This pattern of expression closely parallels cyclins A and D1 in these cells. These data indicate that in cycling normal mammary epithelial cells, p21 is present throughout the cell cycle, with the exception of late G₂ and mitosis. In addition, p21 levels are low in growth factor deprived cells compared with cycling cells. We have not examined whether p27 is cell cycle regulated in these cells as p27 protein is undetectable using the available antibodies and the mRNA is 15-20 fold lower for p27 than for p21 in these cells.

Expression of important cell cycle regulators are often altered in tumor cells. Several instances of cyclin gene alteration and overexpression have been documented in tumors, consistent with the positive role of cyclins in cell growth. Therefore, one of the original goals of our research was to determine whether p21 expression was altered in tumor cells. Keyomarsi and Pardee (56) performed an extensive analysis of cell cycle regulator expression in 10 independent cell lines derived from human breast cancer tissue and discovered alterations in cyclin E in all lines examined in addition to perturbation of other regulators. Together with Dr. Keyomarsi, we also examined the abundance of p21 mRNA using both normal breast epithelial cells and these same 10 cell lines derived from tumor tissue (Figure 1C). In each of three normal strains examined, high p21 message levels were found, while in all tumor cell lines, p21 mRNA was either reduced or absent. Interestingly, the five cell lines showing the greatest reduction in p21 expression also lack estrogen responsiveness (56). An additional initial goal of our work was to determine whether p53 was involved in regulating p21 expression in the mammary gland. Using *in situ* hybridization during mouse development, we were unable to identify any differences in p21 expression in p53-knockout vs. wt mice (49), suggesting that p53 does not control the basal level of p21 expression in a wide array of cell types. Because the p53 status of many of the

breast cancer cell lines used in the experiment in Figure 1C is known, we can make inferences concerning the question of whether p53 is regulating p21 expression in the breast. Many of the cell lines which still express p21 (i.e. lanes 6 and 8) have mutant p53. Therefore it is unlikely that p53 is required for p21 expression in these cells.

A number of laboratories have examined a large number of multiple tumor types, including breast tumors, for mutations in the p21 gene. With the exception of prostate where 2 potential inactivating mutations have been identified, there is a scarcity of mutations in the p21 gene (reviewed in 48). This indicates that p21 is not a critical mutational target in breast but does not exclude the possibility that its activity and or abundance is not affected by oncogenic mechanisms. Recent studies found that p27 levels are affected in breast cancer and that the combination of p27 levels and cyclin E levels is an excellent predictor of prognosis in breast cancer (57, 58). This being the case, it is important to determine whether p21 expression is also altered in breast cancers. We initially planned to collaborate with E. Liu who was director of the University of North Carolina SPORE for breast cancer to look at the expression of p21 in normal breast tissues and breast cancer samples. However, because Dr. Liu moved to the NIH, these studies were put on hold by those in charge of the UNC SPORE. In the mean time, we have arranged to obtain samples locally through The Methodist Hospital, a Baylor College of Medicine affiliate. The samples have been analyzed by a pathologist, Dr. Thomas Wheeler, and include invasive carcinoma and DCIS. To facilitate these studies, we have generated monoclonal antibodies against p21 which work for immunohistochemistry (see below). These studies will continue through the final year of the grant. In this regard, we have recently been in communication with Dr. Michele Pagano who was involved in the analysis of p27 as a breast cancer prognostic marker. Dr. Pagano has found that approximately 50% of 56 mammary tumors have lost expression of p21. Of these, half are p53 positive and half are p53 negative. He also found that p53+, p21-expression was significantly associated with high tumor grade. These are interesting preliminary results and we will extend these to additional samples.

Aim 5: Analysis of p21 and associated proteins

The goal of aim 5 was to analyze p21 associated proteins, to explore p21 regulation by phosphorylation and other mechanisms, and to determine which Cdks are inhibited by p21. Most of these aims have been accomplished (see Table 1) and several papers have already appeared. These studies will not be discussed here since they were the focus of previous progress reports.

A major goal of our work was to develop monoclonal antibodies against p21. This was for several reasons, including having highly specific reagents for western blotting and immunohistochemistry (see aim 4). In addition, it is frequently the case that particular monoclonal antibodies will immunoprecipitate particular forms of the protein or its complexes. Thus, a panel of monoclonal antibodies provides a set of reagents for looking at complexes formed in vivo and potentially allows a subset of p21 containing complexes to be studied independent of other p21 containing complexes.

Production of monoclonal antibodies against CIP/KIP family members. Because of the relatively high degree of sequence identity among CIP/KIP proteins, particularly in the inhibitory domain, there is potential for antibody crossreaction. We have noted substantial crossreaction of some polyclonal anti-p27 antibodies with both p21 and p57 (J.W.H., unpublished data). Therefore, when applications involve either immunoprecipitation or immunohistochemistry, it is important to verify that the antibody used is highly specific. Some, but not all, monoclonals may have advantages in this regard. In this section, we describe the preparation and characterization of monoclonal antibodies

against p21 which was done in collaboration with Dr. Ed Harlow and Dr. Brian Dynlacht (59). In a independent study, we also generated a similar panel of antibodies against p57 which facilitates comparisons between these two CKIs (59).

p21 Antibodies. In generating p21 antibodies, we chose to use antigen that was purified from the soluble fraction of *E. coli*. In principle, this may generate antibodies that recognize properly folded forms of the protein and therefore may be more applicable to immunoprecipitation procedures. Mice were injected a total of seven times every three to four weeks intraperitoneally with approximately 25 µg of p21 per injection (purified through a HiTrap SP column). Intravenous injection (with 30 µg of protein) and fusion were performed using standard procedures.

Antibodies were screened initially by ELISA, with 0.3 µg of purified p21 per well. Of the 800 colonies whose culture supernatants were screened, 80 were positive. ELISA-positive antibodies were then tested in three other assays: western blotting, immunoprecipitation, and coupled immunoprecipitation/kinase assays. Thirty-five of the 80 ELISA positives were also positive by western blot analysis. In addition, the strongest (as well as some weaker) ELISA positives were screened by immunoprecipitation of ³⁵S-methionine labeled extracts of insect cells infected with a baculovirus expressing p21. In these extracts, p21 represents a small percentage of the total input proteins, so this is a stringent assay for specificity of immunoprecipitation. Of 39 ELISA positives screened in this way, 27 were positive as judged by immunoprecipitation. Two final criteria were used to determine which antibodies should be clonally selected. First, antibodies that were positive by the latter method were assayed for the ability to immunoprecipitate cyclin-dependent kinase activity from WI-38 whole cell lysates. Although the physiological relevance, if any, is unknown, it has been shown previously that p21 immune complexes from WI38 fibroblasts or from insect cell co-infections contain significant quantities of histone H1 kinase activity (52, 60). Nineteen of 24 antibodies were capable of immunoprecipitating varying degrees of kinase activity (Table 2).

Based on these analyses, 13 hybridoma wells were subjected to single cell cloning and positive clones used to produce antibody supernatants for further characterization. Monoclonal antibodies were tested for relative reactivity and specificity by immunoblotting of WI-38 whole cell lysates as well as bacterial CIP/KIP proteins, immunoprecipitation of p21 with and without associated kinases, and some antibodies were tested for use in immunohistochemistry. Finally, antibodies were epitope mapped by screening them against immunoblots with GST fusion proteins containing different portions of p21. These included fusion proteins containing full-length p21, residues 70-164, 1-80, and 70-100. Epitopes recognized by the antibodies were subsequently mapped more finely with smaller portions of p21. A summary of the properties of the resulting 13 monoclonal antibodies is shown in Tables 2 and 3.

Summary of anti-p21 antibody properties. The anti-p21 monoclonal antibodies were grouped into six categories based on epitope mapping experiments and immunoprecipitation of endogenous p21 complexes from metabolically labeled WI-38 fibroblasts (see Table 2-3). To test each antibody, whole cell extracts of these cells were immunoprecipitated using established procedures, and western blots of the immunoprecipitates were probed sequentially with the antibodies listed in Table 3. Interestingly, the anti-p21 antibodies immunoprecipitated different subsets of proteins in complexes with p21, and the pattern of polypeptides observed in such complexes correlated to some extent with the epitope recognized by the antibody (Table 2). For example, antibodies recognizing amino-terminal or carboxy-terminal portions of p21 were able to immunoprecipitate the full complement of proteins previously identified in p21 complexes,

including cyclins A, D, and E, as well as PCNA, Cdk 2 and Cdk4. Antibodies CP2 and CP68 belong in this class. In contrast, another class of antibodies (exemplified by CP36) was restricted in its ability to precipitate only cyclin A complexes with Cdk2. A third class (CP55) was able to immunoprecipitate p21 to the exclusion of all other previously identified proteins. Consistent with this, CP55 has been shown to significantly reduce the activity of p21 as an inhibitor of cyclin A/Cdk2.

The specificity of these antibodies toward different kinase complexes was confirmed by immunoprecipitation of complexes produced by baculovirus infection of insect cells using different combinations of Cdk and cyclin viruses (Figure 2). Briefly, insect cells were infected with viruses expressing cyclin A, Cdk2, and p21 or cyclin E, Cdk2, and p21 and cell lysates used for immunoprecipitation with three monoclonal antibodies displaying differential specificity with WI38 lysates (Table 2). Mab419 (directed against p107) was used as a negative control. Mab68 immunoprecipitated both cyclin A/Cdk2 and cyclin E/Cdk2 while Mab74 brought down cyclin A/Cdk2 but not cyclin E/Cdk2. Mab55 immunoprecipitated only monomeric p21.

CP36 is the strongest antibody identified for immunoblotting and gives approximately 10-fold stronger signals than the other antibodies (Table 2). This antibody recognizes an epitope located between amino acids 17 and 30. This region is highly conserved between CIP/KIP family members and overlaps the cyclin binding domain. We have found that CP36 cross-reacts weakly with recombinant p27 and p57 on immunoblots; signals are 50 and 100-fold weaker than with an equal quantity of p21, respectively. In addition, CP36 can immunoprecipitate p27 and p57 but with very low efficiency. None of the other antibodies have been found to cross-react with other CIP/KIP family members. In addition, despite the high degree of conservation between human and rodent CIP/KIP proteins, none of the anti-p21 antibodies reacts strongly with the mouse protein, although CP36 recognizes rat p21 in immunoblots and in immunohistochemical assays (G. Darlington, personal communication).

These antibodies have been made freely available to investigators in the field and have been useful in the detection of p21 in various settings. We will use these antibodies for the immunohistochemical studies indicated in aim 4.

Phosphorylation of p21 by MAP kinases. One goal of our work is to understand how p21 is regulated. One possible mode of regulation is by phosphorylation. As we reported in our previous progress report, the majority of p21 in fibroblasts is in an unphosphorylated form (60). Under some conditions, however, it is possible to see a fraction of p21 with reduced electrophoretic mobility. This fraction is thought to be phosphorylated (60). In vitro, this phosphorylation can be carried out by Cdk2/cyclin A when present in excess over p21 (60).

We have found that p21 is readily phosphorylated by the MAP kinase Erk2 (Figure 3). This is interesting in light of the role that Erk2 plays in initiating the cell division cycle after Ras activation. It is possible that phosphorylation of p21 by Erk2 affects p21 function in some way, but any effects are unlikely to be direct. We have found that phosphorylation of p21 by Erk2 does not disrupt association of p21 with Cdks nor does it affect its ability to act as an inhibitor (data not shown).

Recent studies have shown that p27 is destroyed by ubiquitin mediated proteolysis and that p27 destruction requires its phosphorylation. This phosphorylation event can be carried out by excess Cdk2 but it is not clear whether this kinase is the relevant kinase *in vivo*. Interestingly, the consensus sites for Cdks and MAP kinases are similar and it is possible that MAP kinases can phosphorylate similar sites on p21 as that phosphorylated by Cdk2

thereby activating p21 for destruction. p21, like p27, is also thought to be destroyed by the ubiquitin pathway. Although our phosphorylation studies are preliminary, they provide a basis for further study during the final year of the grant involving the regulation of p21 destruction (see Conclusion).

Redundancy among CKIs. Because of the fact that the CIP/KIP proteins are members of a protein family, questions have arisen concerning the redundancy of these proteins in various cell growth control pathways *in vivo*. These questions are perhaps most pronounced in studies of knockout mice where one particular CKI may fulfill the function of the CKI lost by directed deletion of the gene. For example, since p21 knockout mice have no obvious developmental phenotype, it was possible that other CKIs (p27 or p57) were up-regulated in response to p21 loss and that they perform any essential functions of p21 *in vivo*. However, in our previous study, we were unable to find any alterations in the expression of p27 or p57 in p21 knockout mice (51). In a separate study, we have generated mice lacking p57. We examined whether mice lacking p57 compensated for its loss by inducing either p21 or p57. We found no change in the levels of p21 in either muscle or kidney by immunoblotting. While p27 levels were also unaltered in kidney, there was a slight (50%) increase in p27 levels in p57 -deficient mice (see Figure 1 in Zhang et al., in the appendix).

Conclusion:

The last two years has seen a virtual explosion in our understanding of the mechanisms regulating cell cycle progression. Much of this concerns the role of Cdk inhibitors in cell cycle control. Our work funded under this grant involves an analysis of the role of p21 through generation of p21-knockout mice and through biochemical analysis of p21 function. Our approach has been to address patterns of expression during development, to determine whether p21 is required for the p53 checkpoint, tumor suppression, and or development through analysis of mice deficient in p21, and to understand mechanistic aspects of p21 function within the context of other CKIs including p57. Our major contribution has been to demonstrate that p21 is not required for the tumor suppression function of p53 at least in the mouse but that it is involved in G1 checkpoint control. This is an important finding since it focuses mechanistic studies on p53 to other pathways which may be important for tumor suppression such as apoptosis.

There are a number of outstanding questions related to p21 function and regulation. In the fourth year of this grant, we will address the question of whether p21, like p27, displays altered expression in breast cancer using immunohistochemical approaches. If we find reduced or altered expression of p21, as found with p27, then it would be possible that a common mechanism is employed to alter the levels or locations of these two CKIs. We will also pursue the question of how p21 levels are regulated. Based on the data described above, N76 cells display cell cycle regulated p21 abundance, with the protein being destroyed during the G2/M phase of the cell cycle. In fact, the pattern of expression closely parallels that found with cyclin A, which is known to be destroyed by the ubiquitin-proteosome pathway in G2/M. We plan to confirm preliminary reports that p21 is regulated by the ubiquitin pathway and whether this requires its phosphorylation. One setting in which this can be approached is to analyze if the kinetics of destruction of p21 are different during G2/M and other phases of the cell cycle in N76 cells where we see an abrupt reduction in the levels of p21 protein during G2/M (Figure 1E). It may also be possible to examine p21 ubiquitination *in vitro* using extracts from these cells in different cell cycle phases. In this regard, my lab has recently established an *in vitro* system for ubiquitination of the budding yeast CKI p40SIC1 (63). SIC1 ubiquitination absolutely requires that Sic1 be phosphorylated, a parallel found with both p27 and possibly p21 as we have found that p21 is phosphorylated by Erk2 and others have found that it is phosphorylated by excess Cdks.

SIC1 ubiquitination also requires a complex of 4 proteins Cdc4, Skp1, Cdc53, and the ubiquitin conjugating enzyme Cdc34. Together, Cdc53/Skp1/Cdc4 complexes function as an E3 to recognize phosphorylated SIC1 and the E2 Cdc34. It is conceivable that a similar complex exists in mammalian cells to recognize and ubiquitinate p21 and p27. We will explore this possibility through biochemical analysis of Skp1 complexes in mammalian cells.

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Figure Legends

Figure 1.

Tissue distribution, cell cycle regulation, and expression in normal and tumor cell lines of p21.

(A) A Northern blot of mRNA taken from adult human tissues probed with *p21CIP1* cDNA.

The identity of the tissue is indicated above each lane.

(B) Cells (70N) were synchronized by growth factor deprivation for 24 hours and then stimulated to enter the cell cycle by addition of growth factors. Cells were harvested, at the indicated times after re-stimulation and total RNA was prepared. 20 μ g of RNA per lane were fractionated on a formaldehyde-1% agarose gel, transferred to Nytran, and probed with *CIP1* and 36B4 as a loading control.

(C) Northern blot analysis of *p21CIP1* expression on normal human breast cells established from reduction mammoplasty surgical specimen and cells from human breast tumors. Lanes 1-3 contain normal human breast cells from reduction mammoplasty and lanes 4-13 are tumor cell lines with their origins in parentheses: lane 1, 70N, lane 2, 81N, lane 3, 76N, lane 4, MCF-7 (adenenocarcinoma), lane 5, MDA-MB-157 (carcinoma), lane 6, MDA-MB-231 (adenenocarcinoma), lane 7, MDA-MB-436 (adenenocarcinoma), lane 8, T-47D, (ductal carcinoma), lane 9, BT-20 (carcinoma), lane 10, HBL 100 (tumor, breast milk), lane 11, Hs-587T (ductal carcinoma), lane 12, SKBR-3 (adenocarcinoma), lane 13, ZR75T (ductal carcinoma).

(D) p21 protein levels during cell cycle entry from quiescence in normal mammary epithelial cells. Cells were synchronized at released as described in Figure 1A. Protein extracts were made and 50 μ g of protein for each time point subjected to SDS-PAGE and immunoblotting with anti-p21 or anti-cyclin A antibodies.

(E) Expression of p21 during the cell cycle in epithelial cells. 76N or MCF-10-A cells were synchronized by double thymidine block (dT) and growth factor deprivation (GFD), respectively, and whole cell extracts isolated at the indicated times. Fifty micrograms of protein/lane was analyzed by immunoblotting with affinity purified rabbit polyclonal antibodies against p21. Blots were stripped and reprobed with anti-cyclin A and anti-cyclin D1. In 76N cells, flow cytometry demonstrated a peak in G2 DNA content between 6 and 9 h and accumulation of cells with a G1 content at 15-18 h. For MCF-10-A cells S-phase was detected at 20 h while G2 DNA content was detected at 32 h. Detection was accomplished using ECL (Amersham). For GFD (growth factor deprivation), lane numbers 1-12 refer to 0, 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, and 44 h after growth factor addition. For double thymidine block, lane numbers 1-12 refer to 0, 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, and 33 h after release from the block.

Figure 2. Differential recognition of p21 containing complexes by anti-p21 monoclonal antibodies. Insect cells were infected with the indicated combinations of baculoviruses expressing cyclin A, cyclin E, Cdk2, or p21. Forty hr post infection, lysates were made and used in immunoprecipitation reactions with Mab55, Mab68, and Mab74 against p21 and Mab419 against p107 as a negative control. Washed immune complexes were separated by SDS-PAGE and immunoblotted with the indicated antibodies against Cdk2, cyclin A, cyclin E, or p21. Detection was accomplished by ECL (Amersham).

Figure 3. Phosphorylation of p21 by Cdk2/cyclin A and the Map kinase Erk2. p21 (1 μ M; purified from *E. coli*) was incubated with either 1.2 μ M Gst-CyA/Cdk2 or 100 nM Erk2 in kinase buffer containing 50 μ M 32 P-ATP at 37 °C for 30 min. Reaction mixtures were separated by SDS-PAGE and products visualized by autoradiography. The positions of p21, Gst-CyA, and Erk2 (which all get phosphorylated in the reaction) are indicated.

Table 2: Monoclonal Antibodies Against p21CIP1.

Designation	Isotype	Immunoprecipitation		Immunoblotting ^c	Epitope ^d
		p21 protein ^a	kinase activity ^b		
CP2	IgG2a	+++	+	++	1-17
CP13		++	+	+++	81-164
CP19		++	+	+	1-80
CP23		++	+	++	1-80
CP30		+	-	+	1-80
CP36	IgG1	++	+++	+++	17-30
CP49	IgG2b	+++	+	+	100-164
CP50	IgG2b	+/-	-	+	30-78
CP55	IgG2a	+	NT	+	55-80
CP59		+	+	++	81-164
CP68	IgG2b	+++	+	+	100-164
CP69		++	+	+	1-80
CP74		+	++	+++	1-80

^a Immunoprecipitation of p21 was examined using ³⁵S-methionine labeled p21 generated by expression in insect cells. Immune complexes were subjected to SDS-PAGE and proteins visualized by autoradiography.

^b p21-associated kinase activity in whole cell WI38 extracts was measured as described in ref. 10 using histone H1 as substrate.

^c Detection of p21 by immunoblotting was accomplished using whole cell lysates from WI38 cells (30 µg/lane). Detection was accomplished using enhanced chemiluminescence (ECL; Amersham).

^d Epitope mapping was accomplished using immunoblotting with a panel of GST-p21 fusion proteins.

(Table adapted from our paper Dynlacht et al., 1997)

Table 3: Summary of complexes immunoprecipitated from WI38 cell extracts with anti-p21 antibodies.^a

	Cdk2	cyclin A	cyclin E	cyclin D1	Cdk4	PCNA
CP2, CP66	+	+	+	+	+	+
CP13, CP68	+	+	+	+	+	+
CP19, CP23, CP36 ^b , CP74	+	+	-	-	-	-
CP49, CP59	+	+	+	+	+	+
CP55	-	-	-	-	-	-

^a Whole cell extracts from WI38 fibroblasts were immunoprecipitated with the indicated anti-p21 antibodies and immune complexes subjected to immunoblotting with antibodies against the indicated cyclins, Cdks, and PCNA. Detection was accomplished using ECL. Cyclins A and D were detected with rabbit polyclonal antibodies (W. Harper and L. Zukerberg, respectively). Anti-cyclin E (HE 12), anti-Cdk2 (Santa Cruz), anti-PCNA (Oncogene Science), and anti-Cdk4 antibodies were also used.

^b This antibody has been shown to perform well in immunohistochemical analysis of p21 expression in both paraffin and frozen tissue sections.

(Table adapted from Dynlacht et al., 1997)

Figure 1

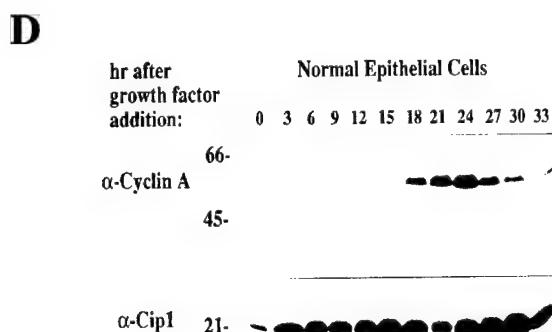
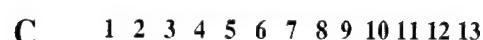
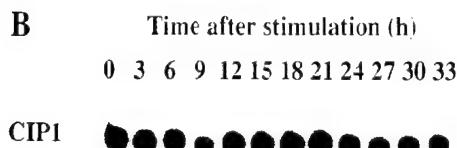
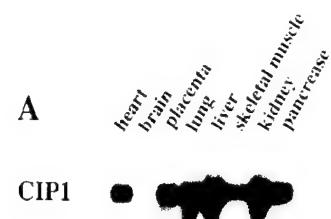


Figure 2

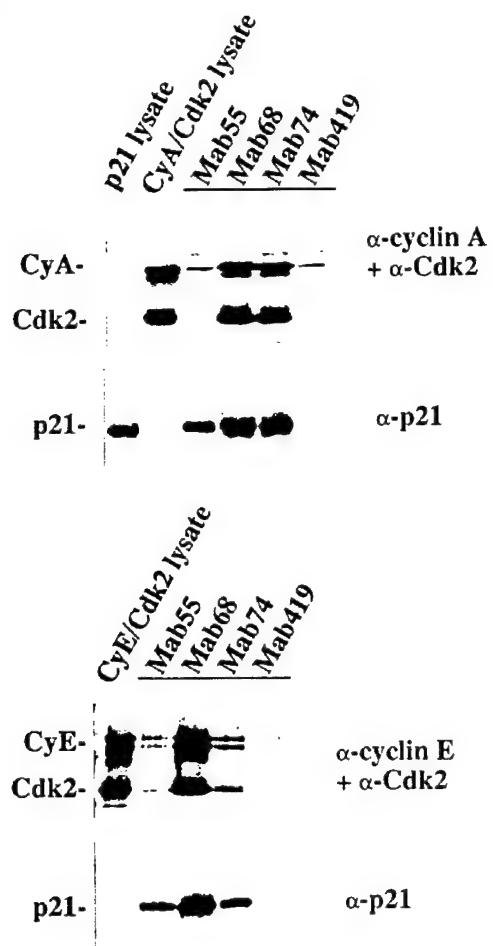
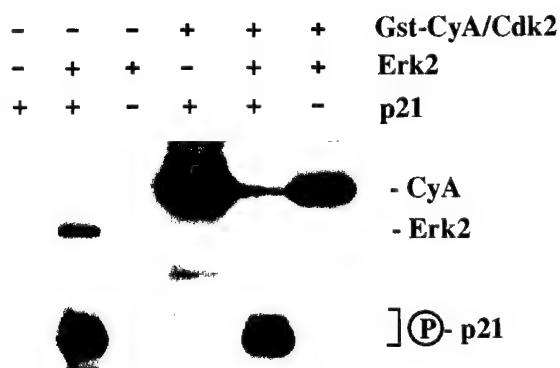


Figure 3



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Publications

Elledge, S.J., Winston, J., and Harper, J.W. (1996) A question of Balance: The roles of cyclin kinase inhibitors in development and tumorigenesis. *Trends in Cell Biology* 6, 388-392.

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Mice Lacking p21^{CIP1/WAF1} Undergo Normal Development, but Are Defective in G1 Checkpoint Control

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Summary

p21^{CIP1/WAF1} is a CDK inhibitor regulated by the tumor suppressor p53 and is hypothesized to mediate G1 arrest. p53 has been suggested to derive anti-oncogenic properties from this relationship. To test these notions, we created mice lacking p21^{CIP1/WAF1}. They develop normally and (unlike p53^{-/-} mice) have not developed spontaneous malignancies during 7 months of observation. Nonetheless, p21^{-/-} embryonic fibroblasts are significantly deficient in their ability to arrest in G1 in response to DNA damage and nucleotide pool perturbation. p21^{-/-} cells also exhibit a significant growth alteration in vitro, achieving a saturation density as high as that observed in p53^{-/-} cells. In contrast, other aspects of p53 function, such as thymocytic apoptosis and the mitotic spindle checkpoint, appear normal. These results establish the role of p21^{CIP1/WAF1} in the G1 checkpoint, but suggest that the anti-apoptotic and the anti-oncogenic effects of p53 are more complex.

Introduction

The ability to control precisely the order and timing of cell cycle events is important for the maintenance of genomic stability and the prevention of mutations that can disrupt normal growth control (reviewed by Hartwell, 1992; Hartwell and Kastan, 1994). The timing of cell cycle progression is regulated through controls called checkpoints. Checkpoints also monitor the physical integrity of DNA and coordinate cell cycle transitions. For example, in response to DNA damage, cells coordinately arrest cell cycle progression and induce the expression of gene products that facilitate DNA repair. Arrest in G1 is thought to prevent replication of damaged genetic templates, and arrest prior to M allows cells to avoid segregation of defective chromosomes.

Primary among mammalian checkpoint genes is the tumor suppressor p53 (reviewed by Hinds and Weinberg,

1994; Cox and Lane, 1995). p53 mutations are the most commonly observed genetic lesion found in human neoplasia (Hollenstein et al., 1991). Mice deficient in p53 develop normally, but are susceptible to spontaneous tumors such that >70% develop tumors by 6 months of age (Donehower et al., 1992; Jacks et al., 1994; Williams et al., 1994; Harvey et al., 1994). p53 is required for arrest in G1 in response to DNA damage such as ionizing radiation (IR) and to perturbation of nucleotide pools (Kastan et al., 1991, 1992; Kuerbitz et al., 1992; Livingstone et al., 1992; Yin et al., 1992). In addition, p53 is required for optimal apoptosis induced by DNA damage (Clarke et al., 1993; Lotem and Sachs, 1993; Lowe et al., 1993) and other agents (Canman et al., 1995). Recently, p53 has been implicated in a spindle checkpoint that controls the ploidy of cells (Cross et al., 1995). Consequently, cells lacking p53 display a striking degree of genomic instability, showing high rates of aneuploidy and gene amplification (Livingstone et al., 1992; Yin et al., 1992).

p53 is a transcription factor (reviewed by Hinds and Weinberg, 1994; Cox and Lane, 1995). In response to genotoxic stress such as DNA damage, p53 protein levels increase by a poorly understood mechanism (Maltzman and Czyzak, 1984), and this alteration in p53 is thought to result in transcription of target genes that mediate its many functions. Among its transcriptional targets are Mdm-2, a negative regulator of p53 thought to be involved in adaptation (Barak et al., 1993; Wu et al., 1993); cyclin G, of unknown function (Okamoto and Beach, 1994); Bax, a protein that promotes apoptosis (Miyashita and Reed, 1995); GADD45, a protein involved in DNA repair (Smith et al., 1994); and p21^{CIP1/WAF1} (El-Deiry et al., 1993), an inhibitor of G1 cyclin-cyclin-dependent kinase (CDK) complexes (Harper et al., 1993; Xiong et al., 1993; Gu et al., 1993), proliferating cell nuclear antigen (Flores-Rozas et al., 1994; Waga et al., 1994), and DNA synthesis (Noda et al., 1994). p21^{CIP1/WAF1} is a member of a related family of CDK inhibitors that include p27^{KIP1} (Polyak et al., 1994; Toyoshima and Hunter, 1994) and p57^{KIP2} (Matsuoka et al., 1995; Lee et al., 1995; reviewed by Elledge and Harper, 1994; Sherr and Roberts, 1995). However, unlike p21^{CIP1/WAF1}, there is no evidence that these are regulated by p53. In the case of p57^{KIP2}, positive regulation by p53 has been ruled out (Matsuoka et al., 1995).

Of the known transcriptional targets of p53, p21^{CIP1/WAF1} is the most likely to control cell cycle arrest. It is induced by DNA damage in a p53-dependent manner and is found associated with inactive cyclin E-CDK2 complexes (Dulic et al., 1994; El-Deiry et al., 1994). Cyclin E-CDK2 complexes have been shown to be essential for the G1 to S phase transition (Ohtsubo et al., 1995). p21^{CIP1/WAF1} can arrest the cell cycle in G1 when overexpressed (Harper et al., 1995). In some fibroblast cell lines, p21^{CIP1/WAF1} basal expression is dependent on p53; however, in embryonic and adult mouse tissues, basal expression is largely independent of p53 (Parker et al., 1995; Macleod et al., 1995). Expression induced by γ -irradiation in vivo is, however,

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p53 dependent (Macleod et al., 1995). p21^{CIP1/WAF1} is expressed during embryogenesis primarily in a subset of cells that are amitotic, thus potentially contributing to cell cycle exit during differentiation (Parker et al., 1995). p21^{CIP1/WAF1} induction has also been observed in cell lines undergoing differentiation or senescence in vitro (Jiang et al., 1994; Steinman et al., 1994; Parker et al., 1995; Halevy et al., 1995; Noda et al., 1994). Therefore, in theory, organisms may utilize p21^{CIP1/WAF1} in many ways: in proliferating cells in G1, it may contribute to cell cycle transition timing or G1 arrest; in S phase cells, it may block proliferating cell nuclear antigen and slow down DNA synthesis to facilitate repair processes; and in development, it may contribute to cell cycle arrest in terminally differentiating cells.

To explore the role of p21^{CIP1/WAF1} in development and as a mediator of p53 function, we have created mice lacking the p21^{CIP1/WAF1} gene by targeted gene disruption. We have determined that p21^{CIP1/WAF1} is responsible for only a subset of known p53 functions. The implications of these findings with respect to tumorigenesis are discussed.

Results

Targeted Disruption of the p21^{CIP1/WAF1} Gene Creates a Null Mutation

The targeting construct p21^{neo} (Figure 1A) was used to delete exon 2 of the p21^{CIP1/WAF1} gene. This exon encodes about 90% of coding sequence of the p21^{CIP1/WAF1} protein (El-Deiry et al., 1993), and its replacement through homologous recombination is predicted to create a null mutation. Of 70 G418 and FIAU double-resistant embryonic stem (ES) cell clones analyzed by Southern blot analysis for homologous recombination, one contained a correct targeting event (Figure 1B). Germline transmission was obtained from the injection of this ES clone into C57BL/6J blastocysts. Southern blot analysis indicated that about 50% of agouti offspring were heterozygous for the targeted mutation (p21^{+/−}).

p21^{CIP1/WAF1} is expressed in most organs and tissues in a p53-independent manner during murine embryonic and postnatal development (Parker et al., 1995; Macleod et al., 1995). To study the possible role of p21^{CIP1/WAF1} in murine development, we intercrossed mice heterozygous for the targeted disruption to produce homozygous offspring. For the sake of brevity, we will refer to the genotype using the abbreviated form, p21⁺ or p21[−]. These crosses gave rise to litters of normal size, with living, apparently normal p21^{−/−} offspring present at a frequency consistent with Mendelian inheritance. p21^{−/−} mice were monitored for evidence of illness or tumor formation weekly for up to 7 months, and peripheral blood examinations, which showed no evidence of red or white blood cell abnormalities, were carried out at 4 months of age. Histological sections from several organs, such as muscle, testis, vertebral bones, and brain, were examined and were found to be normal (data not shown).

Cultured mouse embryonic fibroblasts (MEFs) were derived from day 14 embryos of the three possible geno-

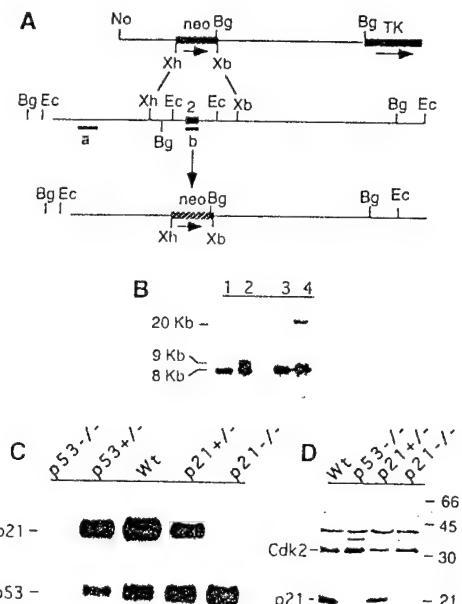


Figure 1. Targeted Disruption of the p21 Gene Produces a Null Mutation

(A) This targeting construct contains a 8 kb p21^{CIP1/WAF1} genomic sequence with a PGK-neo cassette (Tybulewicz et al., 1991), inserted through XbaI (Xb) and Xhol (Xh) sites, that eliminates a 3 kb genomic fragment containing exon 2 of the p21^{CIP1/WAF1} gene. Transcriptional directions of the neomycin (neo) and the thymine kinase (tk) genes are indicated by arrows. Prior to electroporation, the targeting vector was linearized at the indicated unique NotI (No) site. Homologous recombination within the p21 locus would introduce the neo gene and delete the endogenous exon 2 (2). Other abbreviations: Bg, BgIII; Ec, EcoRI; a, probe a; b, probe b.

(B) Southern blot analysis of DNA isolated from parental ES cells (lanes 1 and 3) and a targeted ES cell clone (lanes 2 and 4). As expected, the BgIII restriction fragment size change from 8 kb to 9 kb is seen in the targeted cells (lanes 1 and 2) using the fragment ³²P-labeled, 0.7 kb AccI 5' probe indicated in the insert map (see probe a in [A]). Correct targeting events were confirmed by analysis of the fragments produced following EcoRI digestion and detected using the same probe (lanes 3 and 4).

(C) Northern blot analysis of RNAs isolated from wild-type (WT), p53^{+/−}, p21^{+/−}, and p21^{−/−} MEFs. About 2 μ g of poly(A) RNA was loaded on the each lane. Following the transfer, the filter was first hybridized with the p21^{CIP1/WAF1} probe (see probe b in [A]) to detect expression. After stripping, the filter was hybridized with a p53 cDNA probe (see Experimental Procedures) to detect p53 expression.

(D) Analysis of p21 protein in MEFs derived from control and mutant mice. Whole-cell lysates of MEFs (at passage 5) derived from the indicated mouse genotypes were subjected to SDS-PAGE and immunoblotting as described in Experimental Procedures. The portion of the blot containing proteins of <30 kDa was probed with a monoclonal antibody directed against mouse p21, while the portion of the blot containing proteins of >30 kDa was probed with anti-CDK2 as a loading control. CDK2 mRNA in mouse cells is translated as two protein isoforms of 32 and 40 kDa (Noguchi et al., 1993).

types, wild-type, p21^{+/−}, and p21^{−/−}. These were compared with similarly derived p53^{+/−} and p53^{−/−} MEFs with respect to p21^{CIP1/WAF1} mRNA (Figure 1C) and protein (Figure 1D). Very little p21^{CIP1/WAF1} mRNA (about 2% of wild-type) could be detected in samples from p53-deficient cells (Figure 1C, lane 1). No p21^{CIP1/WAF1} mRNA was detected in samples

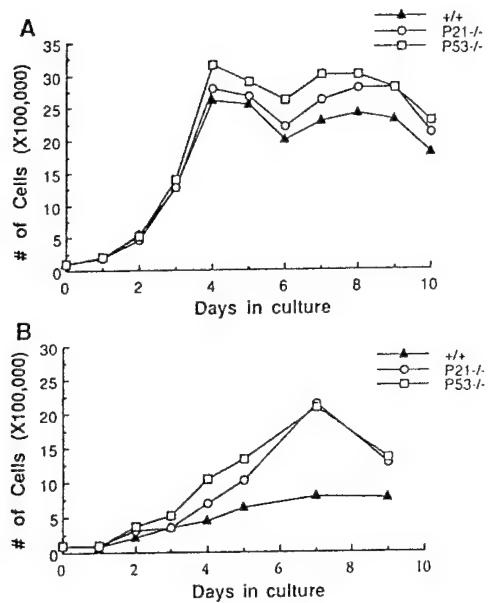


Figure 2. Saturation Densities and Growth Properties of MEFs
(A) Saturation density and growth analyses of MEFs at passage 3. Cells (10^5) from wild-type (+/+) and p21^{-/-} and p53^{-/-} (as indicated in the figure) were plated in replicate 35 mm culture dishes as described in Experimental Procedures. Cell number at each timepoint represents the average of duplicate plates.
(B) Growth curves of MEFs at passage 5 to 6. The genotypes of each cell are indicated in the figure.

from p21^{-/-} cells (Figure 1C, lane 5), nor could p21^{CIP1/WAF1} protein be detected in samples from either p53- or p21-deficient cells (Figure 1D). Given these results and the extensive nature of the deletion, we conclude that this p21^{CIP1/WAF1} mutation is a null.

Growth Properties of p21^{-/-} Cultured MEFs

We examined the initial growth properties of these mutant cells during culture in vitro. Accordingly, proliferation and saturation densities of wild-type, p21^{-/-}, and p53^{-/-} MEFs were analyzed at two passage points. At lower passages (passages 1–3), all of the cells, irrespective of their genotypes, grew quite rapidly and had similar growth rates before becoming confluent (Figure 2A). All MEFs showed contact inhibition, but the monolayers formed by p21^{-/-} and p53^{-/-} MEFs were more crowded than those formed by wild-type cells. In addition, the saturation densities of p53^{-/-} cells were significantly higher than those of wild-type cells, while those of p21^{-/-} cells were intermediate between those of wild-type and p53^{-/-} cells (Figure 2A).

At later passages (passages 5–15), as growth of wild-type cells slowed, differences in the saturation densities between the p53^{-/-} and wild-type MEFs became much more pronounced. p21^{-/-} MEFs behaved more like p53^{-/-} MEFs (Figure 2B). Between passages 5 and 15, proliferation of wild-type MEFs was significantly reduced, as seen by the passage 5 and 6 cells shown in Figure 2B. Wild-type cells gradually accelerated growth rates between passages 15 and 20, while the behavior of p21^{-/-} and p53^{-/-} MEFs did not change (data not shown).

The Role of p21 in p53-Mediated G1 Arrest

In response to DNA damage, mammalian cells arrest the cell cycle. Although the number of points at which the cycle can arrest have not been fully elucidated, it is clear that there is a block to entry into S phase that is mediated in part by arrest in G1. This cell cycle arrest is dependent upon the p53 gene product. Although it is not clear how p53 achieves cell cycle arrest, it has been suggested that it is through the transcriptional activation of the p21^{CIP1/WAF1} gene (El-Deiry et al., 1993; Harper et al., 1993). To examine the contribution of p21^{CIP1/WAF1} to the G1 checkpoint, we examined the ability of wild-type and mutant MEFs to reduce the population of S phase cells in response to γ -irradiation.

Serum-starved cells were irradiated and stimulated to enter the cycle by the addition of serum. Bromodeoxyuridine (BrdU) was added with serum to allow detection of cells entering S phase. Cells were harvested 24 hr later, and the number having entered S phase were determined by bivariate fluorescence-activated cell-sorting (FACS) analysis. Incorporation of BrdU indicates cells in S phase (see the example shown in Figure 3A). As indicated in Figure 3B, wild-type cells showed a 50% reduction in the number of S phase cells relative to unirradiated samples averaged over several experiments. By contrast, p53-defective cells showed nearly complete deficiency in the ability to arrest in G1, with, on average, only 2.8% reduction in response to γ -irradiation, as previously reported (Kastan et al., 1992). p21^{-/-} cells had a phenotype intermediate between wild-type and p53^{-/-} cells, showing, on average, a 20% reduction in cells entering S phase. These results indicate that p21^{CIP1/WAF1} partially mediates the role of p53 in G1 arrest in response to γ -irradiation. In contrast with some cells (Kastan et al., 1992), significantly higher levels of irradiation was required to achieve a significant reduction in S phase cells with our wild-type MEFs. Control experiments with WI38 cells, a normal human fibroblast strain, demonstrated that 5 Gy was sufficient to reduce S phase cells from 38% to 3% (data not shown), controlling for the proper calibration of our radiation source and confirming the increased radioresistance of our MEFs.

In addition to γ -irradiation, cells can arrest in G1 in response to other types of damage or metabolic perturbations. N-(phosphonacetyl)-L-aspartate (PALA) is a specific inhibitor of de novo uridine biosynthesis (Swyryd et al., 1974) that works through inhibition of the CAD gene (Wahl et al., 1979). Cells treated with PALA show a p53-dependent decrease of entry into S phase (Yin et al., 1992; S. Linke and G. Wahl, personal communication). We examined the role of p21^{CIP1/WAF1} in cell cycle arrest in response to PALA. Asynchronous cells were treated with varying amounts of PALA for 48 hr and then labeled for 30 min with BrdU to identify cells in S phase. Both p53^{-/-} and p21^{-/-} cells display a decrease in the G1 population relative to wild-type cells and an increase in S phase cells (Figure 4A).

The large increase in the S phase population is thought to arise from cells that fail to arrest in G1, enter S, and become arrested there owing to a lack of dTTP caused by

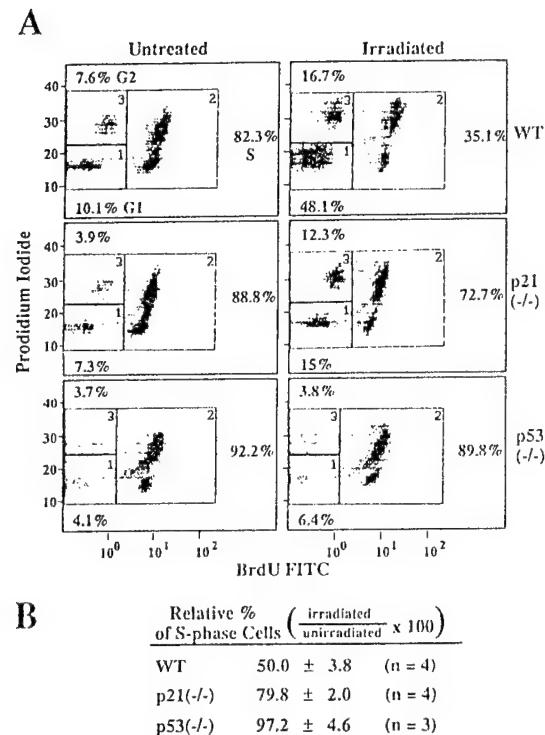
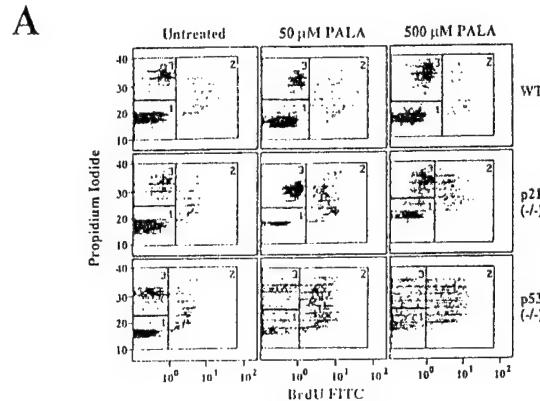


Figure 3. MEF Lacking p21^{CIP1/WAF1} Display a Defect in the Ability to Block S Phase Entry after γ -Irradiation

(A) A representative bivariate flow cytometric analysis of synchronized wild-type (WT), p21^{-/-}, or p53^{-/-} MEFs 24 hr after exposure to 0 or 20 Gy γ -irradiation. Serum-starved cells (4 days in media containing 0.1% FBS) were released into complete media containing BrdU (65 μ M) and immediately irradiated. At 24 hr after release, cells were harvested and stained for DNA content with propidium iodide and for replicative DNA synthesis with a fluorescein isothiocyanate-conjugated anti-BrdU antibody. Boxes labeled 1, 2, and 3 indicate G1, S, and G2/M phase cells, respectively, and the percentages of cells in each phase of the cycle are indicated for each diagram. Approximately 3000 cells were examined in each analysis.

(B) Quantitative analysis of the effects of γ -irradiation on the number of cells entering S phase for wild-type (WT), p21^{-/-}, and p53^{-/-} MEFs. The percentage of cells entering S phase (BrdU⁺) after irradiation relative to unirradiated cells is shown along with standard deviations for replicate experiments; n refers to the number of independent determinations for each strain.

PALA. Interestingly, p21-deficient cells show a significant increase in G2 cells that is not observed in p53-defective fibroblasts. This may indicate a p21-independent, p53-dependent function in G2 arrest in response to PALA treatment. Although more in-depth experiments are required to confirm this possibility, a role for p53 in G2 arrest has been previously reported (Stewart et al., 1995; Aloni-Grinstein et al., 1995). At higher concentrations of PALA, it becomes difficult to measure precisely the S and G2 contents in p21^{-/-} and p53^{-/-} cells, as aberrant patterns of BrdU incorporation are observed in FACS analysis. Nevertheless, it is clear that the p21^{-/-} fibroblasts are defective in the G1 checkpoint activated by nucleotide depletion, thereby implicating p21^{CIP1/WAF1} as a significant mediator of the checkpoint function of p53 in response to nucleotide depletion.



Strain	[PALA]	0	50 μ M	500 μ M
WT	G1	60.1	64.3	58.3
	S	14.5	13.4	6.1
	G2	25.4	22.3	35.6
p21(-/-)	G1	62.7	19.5	29.8
	S	14.7	40.0	24.6
	G2	23.4	40.4	45.5
p53(-/-)	G1	57.8	19.4	23.1
	S	18.6	56.8	44.9
	G2	23.6	23.8	32.0

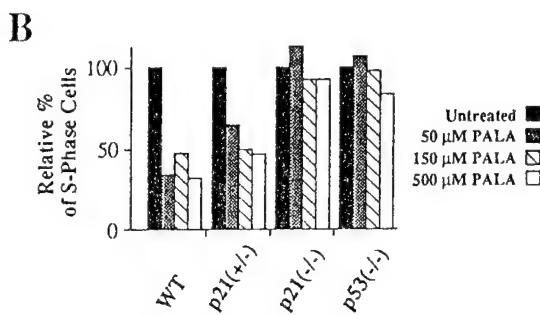


Figure 4. Absence of a Functional PALA-Induced G1 Checkpoint in p21^{-/-} MEFs

(A) Effects of PALA on the cell cycle distribution of asynchronous MEFs. Asynchronous MEFs (passage 3) of the indicated genotypes were treated with various concentrations of PALA for 48 hr. Cells were pulse labeled with BrdU (30 min), harvested, and analyzed for DNA synthesis and DNA content by FACS analysis as described for Figure 3. Below is the quantitation of cell cycle phase distribution for these cells after PALA treatment.

(B) Effects of PALA on S phase entry of synchronized MEFs. The indicated MEF cultures at passages 3 and 4 were serum starved for 84 hr prior to addition of the indicated concentrations of PALA for 12 hr, and then the cells were released into growth media containing the same concentrations of PALA and BrdU (65 μ M). At 24 hr, cells were harvested and subjected to bivariate flow cytometric analysis. The percentage of cells entering S phase (BrdU⁺) in the presence of PALA normalized to the number of cells entering S phase in the absence of PALA is shown. The percentage of cells entering S phase in the absence of PALA was 41%, 32%, 60%, and 82% for wild-type (WT), p21^{-/-}, p21^{+/-}, and p53^{-/-}, respectively.

Interpretation and quantitation of the pulse-labeling results on asynchronous cultures are complicated in part by the fact that cells can arrest in G1, G2, or M and thereby prevent S phase entry. To measure more precisely the

effects of PALA on the G1 to S phase transition, we performed experiments using synchronized cells. Serum-starved cells were stimulated to enter the cycle in the presence of PALA and BrdU by the addition of serum. Cells were harvested 24 hr later, and the number having entered S phase were quantitated by bivariate FACS analysis (Figure 4B). As observed in asynchronous cells, both p21^{-/-} and p53^{-/-} cells show a nearly complete defect in G1 arrest relative to wild-type and p21^{-/-} cells. Unlike the results with γ -irradiation, the defect in PALA-dependent arrest in p21^{-/-} cells appears to be nearly as severe as the defect with p53^{-/-} cells. This difference may be due to the precise position in the G1 to S phase transition that cells arrest in response to the different treatments, to the additional pathways activated by the stimuli, or to the degree of activation of the p53 protein itself.

p21^{CIP1/WAF1} Is Not Required for the Function of the p53-Dependent Mitotic Spindle Checkpoint

In addition to the G1 checkpoint, p53 controls a mitotic spindle checkpoint (Cross et al., 1995). Wild-type fibroblasts treated with the microtubule inhibitor colcemid arrest with a 4N DNA content. However, cells defective for p53 fail to arrest and proceed through the cell cycle, becoming polyploid. Wild-type, p21^{-/-}, and p53^{-/-} fibroblasts were examined for their ability to arrest the cell cycle in response to colcemid (Figure 5). While p53^{-/-} cells fail to arrest in the presence of colcemid and accumulate substantial quantities of polyploid cells, p21^{-/-} and wild-type cells have intact mitotic checkpoints.

In the absence of spindle inhibitors, 50% of p53^{-/-} MEFs became tetraploid at passage 7 (Cross et al., 1995), and nearly all p53^{-/-} MEFs became tetraploid or polyploid at passage 9 (Livingstone et al., 1992). To determine whether p21^{CIP1/WAF1} is involved in maintaining the chromosomal ploidy during culture, we determined chromosome numbers for wild-type, p21^{-/-}, and p53^{-/-} MEFs at passages 3 and 15. The chromosome number was obtained by counting 50 intact metaphase spreads of each cell type. At passage 3, wild-type and p21^{-/-} MEFs had a similar karyotype; i.e., 90% of cells had a typical diploid chromosomal number of 40 and <10% cells were tetraploid. In contrast, 30% of p53^{-/-} cells became tetraploid or polyploid, and an additional 10% contained chromosome numbers between 62 and 78. At passage 15, 30% of wild-type and 40% of p21^{-/-} MEFs had 40 chromosomes, whereas >90% of the p53^{-/-} MEFs were aneuploid, tetraploid, or polyploid. Thus, p21^{CIP1/WAF1} is not required for the operation of the mitotic spindle checkpoint.

p21^{CIP1/WAF1} Is Not Required for p53-Dependent Apoptosis

Thymocytes readily undergo apoptosis after DNA damage in a p53-dependent manner (Lowe et al., 1993; Clarke et al., 1993). To determine whether p21^{CIP1/WAF1} is required for this process, we isolated thymocytes from wild-type, p21^{-/-}, and p53^{-/-} mice and determined the percentage of apoptotic cells after γ -irradiation. As shown in Figure 6A, wild-type and p21^{-/-} thymocytes show a dramatic increase of percentages of cells undergoing apoptosis (from

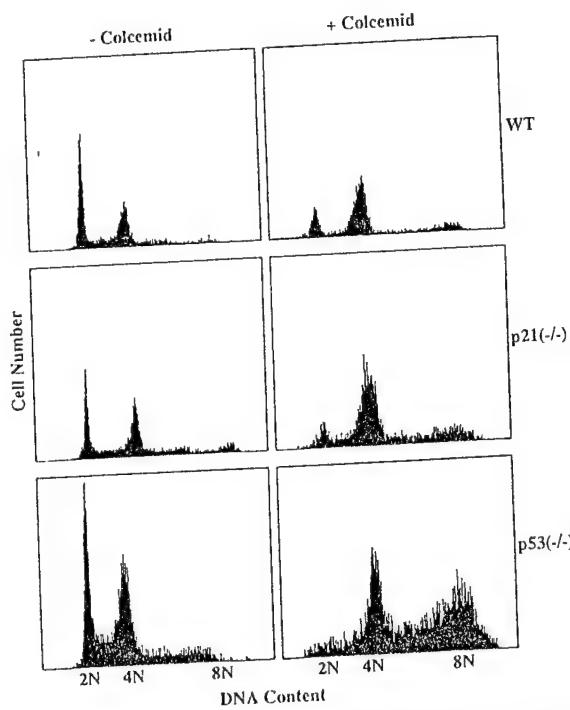


Figure 5. The p53-Dependent Spindle Checkpoint Induced by Colcemid Is Functional in p21^{-/-} MEFs
Asynchronous wild-type (WT), p21^{-/-}, and p53^{-/-} MEFs at passage 3 were incubated in the absence or presence of 500 ng/ml colcemid for 24 hr. Cells were harvested and the DNA content determined by flow cytometry after DNA staining with propidium iodide. DNA content flow cytometric histograms are shown, as are the positions of peaks for 2N, 4N, and 8N DNA content. At least 3000 cells were examined in each experiment.

about 10% to 60%), whereas the p53^{-/-} thymocytes show only a slight increase of apoptotic cells. Cell death by apoptosis was confirmed by gel electrophoretic analysis of genomic DNA (Figure 6B). The DNA fragmentation patterns displayed by wild-type and p21^{-/-} thymocytes were prominent and indistinguishable, while that of the p53^{-/-} thymocytes was greatly reduced by comparison. Thus, it appears that p53-dependent apoptosis in mouse thymocytes does not require p21^{CIP1/WAF1}.

Absence of Accelerated Tumorigenesis

in p21^{-/-} Mice

p53-deficient mice display a marked and rapid rate of early tumorigenesis (Donehower et al., 1992; Jacks et al., 1994). Since p21^{CIP1/WAF1} is regulated by p53 and is involved in the G1 DNA damage checkpoint, it is conceivable that p21^{-/-} mice would also display increased rates of tumorigenesis. To date, we have carefully followed 12 p21^{-/-} mice who are now >7 months of age (and >20 mice now between the ages of 3 and 4 months). In contrast with p53^{-/-} mice, >70% of which develop tumors by 6 months of age (Donehower et al., 1992), none of the p21^{-/-} mice developed malignancies, indicating that p53-dependent suppression of early tumor formation does not require p21^{CIP1/WAF1}.

Obviously, these p21^{-/-} mice will be followed into old age, and a propensity for late tumorigenesis may emerge.

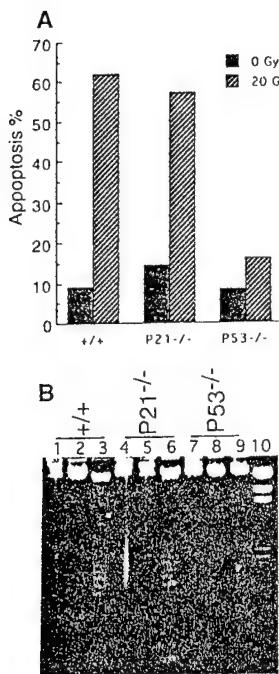


Figure 6. Induction of Apoptosis in $p21^{-/-}$ Thymocytes

(A) Percentages of thymocytes that undergo apoptosis after irradiation at 0 or 20 Gy. After irradiation, the wild-type (+/+) and $p21^{-/-}$ cells were cultured at 37°C for 8 hr before analysis by FACS (Telford et al., 1994).

(B) Gel electrophoresis of genomic DNA isolated from wild-type (+/+) and $p21^{-/-}$ thymocytes with and without γ -irradiation. Lanes 1, 4, and 7 were DNA isolated at time 0 without IR treatment. Lanes 2, 5, and 8 were DNA isolated at 8 hr without IR treatment. Lanes 3, 6, and 9 were DNA isolated at 8 hr after IR treatment. Lane 10 was λ DNA digested by HindIII.

Moreover, in view of the effect that $p21^{CIP1/WAF1}$ has on the G1 DNA damage checkpoint, it will be important to assess the radiation sensitivity of these mice. Further, since we find an even greater sensitivity to tumorigenesis as we breed the $p53^{-/-}$ allele into an inbred FVB genetic background (all of >30 $p53^{-/-}$ mice followed developed tumors before the age of 6 months [C. D. and P. L., unpublished data]), it will be of interest to follow these mice as the $p21^{-/-}$ mutation is passed into various inbred backgrounds in an effort to discover modulating genes. Moreover, in view of the possibility that other members of the p21 class of CDK inhibitors might support functions in which p21 normally participates, it is worth noting that preliminary experiments indicate that mRNAs corresponding to the related CDK inhibitors p27 and p57 are present in roughly equivalent amounts in MEFs derived from wild-type and $p21^{-/-}$ mice (data not shown).

Discussion

DNA Damage-Induced G1/S Arrest Is Partially Dependent upon $p21^{CIP1/WAF1}$

An extensive literature has emerged concerning the putative roles of $p21^{CIP1/WAF1}$ in cell cycle control, cancer, and

development. In particular, it has been suggested that $p21^{CIP1/WAF1}$ may mediate some or all of the known functions of p53. This work provides direct evidence that $p21^{CIP1/WAF1}$ is necessary for efficient p53-dependent cell cycle arrest in response to DNA damage and metabolic perturbation and is, therefore, a bona fide effector of p53. In response to PALA, G1 arrest in $p21^{-/-}$ cells appears nearly as defective as in $p53^{-/-}$ cells. However, in response to γ -irradiation, the G1 checkpoint is only partially impaired, indicating a second p53-dependent function capable of arresting the cell cycle in G1. The difference between inducing agents may reflect a difference in the extent of p53 activation that could alter which arrest mechanism is activated. Alternatively, the position in the cycle when the PALA signal is sensed might preclude one of the arrest mechanisms.

The nature of the second checkpoint mechanism is unclear but could involve *Gadd45*, a p53-dependent, DNA damage-inducible gene that can arrest the cell cycle when overexpressed (Smith et al., 1994). However, it is not clear that *Gadd45* can arrest the cell cycle under physiological conditions. Alternatively, other mechanisms, such as dephosphorylation of the activating threonine residue (T160 in CDK2), activation of additional CDK inhibitors, or inhibitory phosphorylation, might be used to inactivate CDKs. A fraction of CDK2 is tyrosine phosphorylated on Y15 (Elledge et al., 1992; Gu et al., 1992), and the conserved T14 is also a potential target for inhibitory phosphorylation. These phosphorylations are known to inhibit CDKs. Of course, cells could arrest through a CDK-independent mechanism. Regardless of the nature of the mechanism, the availability of $p21^{-/-}$ cells will facilitate its elucidation.

$p21^{CIP1/WAF1}$ is not absolutely required for contact inhibition or arrest in G0 upon serum starvation. However, it does appear to mediate the effects of p53 on serum dependency, growth kinetics, and arrest density of fibroblasts grown in vitro. Unlike these functions and the G1 checkpoint, both the apoptotic response and the mitotic spindle checkpoint function normally in $p21^{-/-}$ cells. An understanding consistent with our results is summarized in the diagram shown in Figure 7 in which $p21^{CIP1/WAF1}$ accounts for only a subset of known p53 functions.

Role of p21 in Terminal Differentiation

$p21^{CIP1/WAF1}$ expression during mouse development correlates with terminally differentiating tissue such as muscle, outer layers of the epidermis, and olfactory neurons (Parker et al., 1995). It has also been observed to be induced during differentiation of a number of cell types in vitro (Jiang et al., 1994; Steinman et al., 1994; Parker et al., 1995; Halevy et al., 1995). These observations have led to the hypothesis that $p21^{CIP1/WAF1}$ might contribute to cell cycle exit and differentiation. The fact that the $p21^{-/-}$ mice appear to have normal development, both at the gross anatomical and histologic levels, indicates that any essential role for $p21^{CIP1/WAF1}$ in terminal differentiation must be redundant. Kinetic analysis of $p21^{CIP1/WAF1}$ induction in differentiating myoblasts in vitro has revealed that $p21^{CIP1/WAF1}$ is induced at a late stage of differentiation, long after DNA synthesis is blocked (J. W. H., unpublished data), indicat-

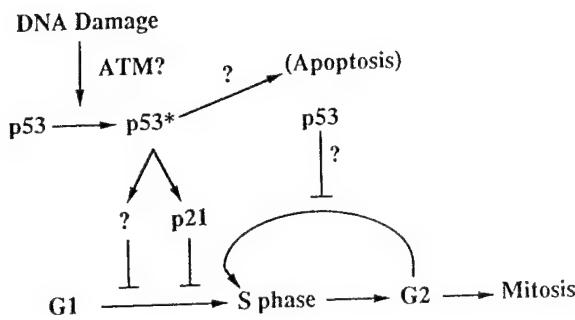


Figure 7. A Current View of the Integrated Actions of p21 and p53
DNA damage leads to stabilization and activation of p53, possibly via the ataxia telangiectasia gene product (Savitsky et al., 1995). The activated p53 protein ($p53^*$) induces transcription of $p21^{CIP1/WAF1}$ and other genes involved in cell cycle arrest and DNA repair. Under certain conditions that are not completely understood, p53 activation can lead to apoptosis. In response to γ -irradiation, p53-dependent cell cycle arrest in G1 functions by both $p21^{CIP1/WAF1}$ -dependent and $p21^{CIP1/WAF1}$ -independent mechanisms that are only partially redundant, while p53-dependent apoptosis in response to γ -irradiation does not require $p21^{CIP1/WAF1}$. In mouse cells, p53 also regulates reentry into S phase when mitosis is blocked, as in the case of the anti-microtubule agents colcemid and nocodazole (Cross et al., 1995). This function is independent of $p21^{CIP1/WAF1}$. p53 may also regulate ploidy in the absence of spindle interference because untreated $p53^{-/-}$ cells rapidly increase ploidy with increased passage.

ing a maintenance rather than causative role. It is not surprising that there would be redundancy in a process of such critical importance to the organism. Indeed, these mice provide a useful background in which to launch a search for the other cell cycle control elements that, along with $p21^{CIP1/WAF1}$, mediate growth arrest and differentiation.

Clarification of the Tumor Suppressive

Effects of p53

One of the attractive aspects of the role of p53 as a tumor suppressor and as an inducer of the G1 cyclin-CDK inhibitor $p21^{CIP1/WAF1}$ was that one could link the function of these proteins and provide a convenient rationale for the ability of p53 to counter the action of oncogenes. For example, an oncogene might promote inappropriate entry into the cell cycle and passage into S phase. The inability of p53 mutants to arrest cells in G1 in response to oncogene action might lead directly to increased proliferation of cells, presumably an important aspect of tumorigenesis. In its simplest form, however, this rationale is incorrect because, unlike $p53^{-/-}$ animals, $p21^{-/-}$ mice do not exhibit a propensity for early tumorigenesis. Indeed, this finding nicely explains the failure to find mutations in the $p21^{CIP1/WAF1}$ gene in human tumors (Shiohara et al., 1994).

There are several plausible explanations for the absence of tumors in $p21^{-/-}$ mice. First, the role of $p21^{CIP1/WAF1}$ in the G1 checkpoint might be partially redundant, as suggested by its intermediate effect on the irradiation checkpoint. Thus, $p21^{-/-}$ cells might show an intermediate increase in genomic instability relative to p53-deficient cells. Such reduced effects in $p21^{-/-}$ cells relative to $p53^{-/-}$ cells can easily be translated into altered mutation frequencies

that, when multiplied over several events, could profoundly influence the overall frequency of tumorigenesis. Alternatively, it is possible that other p21-independent p53 functions, such as apoptosis, may play a more significant role in tumorigenesis than the G1 checkpoint. Several oncogenes, such as *Bcl2* (Strasser et al., 1993), specifically inhibit apoptosis, lending strong support to the notion that the role of p53 in control of apoptosis is important for its tumor-suppressing activity. Finally, it is possible that these other functions in combination with a checkpoint deficiency are responsible for the tumor-suppressing role of p53 *in vivo*.

Experimental Procedures

Targeting Vector

Recombinant phage containing genomic DNA of the p21 locus were isolated from a 129 mouse library (Stratagene) by using a 454 bp fragment containing the second exon of the mouse $p21^{CIP1/WAF1}$ gene as a probe. This fragment was amplified by PCR using a 5' oligonucleotide (5'-TCTTCTGTTCAGCCACAGGC-3') and a 3' oligonucleotide (5'-TGTCAAGGCTGGTCTGCCTCC-3') (El-Dairy et al., 1993). To construct the $p21^{neo}$ targeting vector, we subcloned a 6 kb $BgIII$ -XbaI fragment that is 3' to the second exon of the $p21^{CIP1/WAF1}$ gene into Bam HI and XbaI sites of pPNT (Tybulewicz et al., 1991). The resulting construct was cleaved with Xba I and Not I, followed by insertion of a 2 kb Xba - Not I fragment (the Not I site is from polylinker of the phage vector) that is 5' to the second exon of the $p21^{CIP1/WAF1}$ gene. The finished construct, $p21^{neo}$, is shown in Figure 1A.

Homologous Recombination In ES Cells and Generation of Germline Chimeras

TC1 ES cells, developed by C. D., T. Wynshaw-Boris, and P. L. (unpublished data), were transfected with Not I-digested $p21^{neo}$ and selected with G418 and FIAU. The culture, electroporation, and selection of ES cells were carried out as described previously (Deng et al., 1994). ES cell colonies that were resistant to both G418 and FIAU were picked and analyzed by Southern blotting for homologous recombination events within the $p21^{CIP1/WAF1}$ locus. Genomic DNAs from these clones and the parental TC1 cell line were digested with $BgIII$ and Eco RI, respectively, followed by the Southern blot transfer analysis using a 0.7 kb Acc I fragment 5' to the targeting vector (Figure 1B).

ES cells heterozygous for the targeted mutation were microinjected into C57BL/6 blastocysts to obtain germline transmission. The injected blastocysts were implanted into the uteri of pseudopregnant Swiss Webster (Taconic) foster mothers and allowed to develop to term. Male chimeras (identified by the presence of agouti coat color) were mated with NIH Black Swiss females (Taconic). Germline transmission was confirmed by agouti coat color in the F1 animals, and all agouti offspring were tested for the presence of the mutated $p21^{CIP1/WAF1}$ allele by Southern blot analysis using the same conditions for the detection of the homologous recombination event in the ES cells.

Northern Blot

RNA was isolated using RNA Tel-60 based on the protocol suggested by the manufacturer (Tel-Test, Incorporated). Poly(A) RNA was prepared using a kit purchased from Pharmacia. About 2 μ g of poly(A) RNA from each sample was electrophoresed on the 1% agarose gel and transferred to a GeneScreen filter (New England Nuclear). The filter was then hybridized with the ^{32}P -labeled probes specific for the p21 and p53 genes. The probe for $p21^{CIP1/WAF1}$ is the 454 bp fragment described earlier, and the p53 probe is a 1.2 kb Eco RI-XbaI fragment containing full-length p53 cDNA (Johnson et al., 1991).

Cell Cultures and Treatments

Primary MEFs were obtained from embryos 14 days after conception that were either wild-type, $p21^{+/+}$, $p21^{-/-}$, $p53^{+/+}$, or $p53^{-/-}$, using established procedures (Robertson, 1987). $p53^{+/+}$ and $p53^{-/-}$ MEFs were derived from embryos obtained by intercrossing mutant mice created

by Donehower et al. (1992). Genotypes for each cell line were verified by Northern and Southern blot analysis. WI38 normal diploid fibroblasts were from the American Type Culture Collection (CC1 75) and were used at passage 20. Cells were cultured at 37°C (5% CO₂) in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) supplemented with penicillin and streptomycin (growth medium). For G0 synchronization by serum starvation, asynchronous cultures at 70%–80% confluence were washed with phosphate-buffered saline and placed in DMEM containing 0.1% FBS for 96 hr. For γ -irradiation (IR), G0-synchronized cells were trypsinized and suspended in growth medium before IR treatment. Cells were irradiated in suspension at room temperature with a ¹³⁷Cs γ -ray source at a rate of 4.1 Gy per minute (1 Gy = 100 rads) and then replated into growth medium in 10 cm dishes at 40%–60% confluence. For experiments involving colcemid, asynchronous cells were treated with 500 ng/ml for 24 hr prior to analysis. For PALA experiments, asynchronous cells were incubated in DMEM containing 10% dialyzed FBS and the indicated concentrations of PALA for 48 hr prior to harvesting. For synchronous experiments, cells were serum starved for 84 hr, and then PALA was added at the indicated concentrations and incubated for 12 hr prior to release into serum containing PALA and BrdU (as suggested by S. Linke and G. Wahl, personal communication). IR and PALA experiments were performed using cells at passages 2–4. Colcemid experiments were performed using passage 3 cells.

Cell Cycle Analysis

Replicative DNA synthesis and DNA content were analyzed using bivariate flow cytometry according to established procedures (Di Leonardo et al., 1994). In brief, cells were pulse labeled with 10 μ M BrdU (Sigma) for 30 min or were continuously labeled with 65 μ M BrdU, harvested, and fixed in 70% ethanol. Fixed cells were kept at -20°C until analysis. Cells were treated with 0.1 N HCl containing 0.7% Triton X-100, followed by heating at 95°C for 10 min and rapid cooling to denature the DNA. Cells were then incubated with fluorescein isothiocyanate-conjugated anti-BrdU antibodies (Becton-Dickinson) and counter stained with propidium iodide containing RNase (20 μ g/ml). The stained cells were analyzed on EPICS Profile equipment. Cell debris and fixation artifacts were gated out, and G1, S, and G2/M populations were quantitated using EPICS software. In each experiment, similar numbers of events were analyzed.

Protein Analysis

Total cell protein (30 μ g) from the indicated cells was prepared as described previously (Harper et al., 1993) and was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12.5% gel, transferred to nitrocellulose, and probed with either a monoclonal antibody (65) directed against mouse p21^{CIP1/WAF1} (provided by Dr. D. Hill, Oncogene Science) or anti-CDK2 antiserum (Santa Cruz Biochemicals). Proteins were visualized by ECL (Amersham) using exposure times of 2 min for p21^{CIP1/WAF1} and 1 min for CDK2.

Cell Proliferation and Saturation Density Assays

Cell proliferation and maximal cell density (saturation density) determinations of the MEFs at passage 3 were carried out as described elsewhere (Xu et al., 1995). In brief, 10³ cells of each line were plated in DMEM with 10% FBS in a series of 35 mm culture dishes. The cultures were maintained for up to 10 days, with changing media each day. Cells were counted daily with a hemocytometer. The proliferation study was repeated using MEFs at passages 5 and 6, this time using a larger container (60 mm culture dishes) to allow cells more time for growth before confluence.

Induction of Apoptosis in Thymocytes

Thymocytes were isolated from wild-type, p21^{-/-}, and p53^{-/-} mice at age 4–6 weeks in growth medium (see above) and adjusted to a density of 1 \times 10⁶ per milliliter. Of this mixture, 10 ml was aliquoted into each 10 cm dish. For each mouse, five plates were prepared. Thymocytes from one plate were used to isolate DNA at time 0, two plates were unirradiated, and two plates were γ -irradiated with 20 Gy. These were subsequently incubated at 37°C for 8 hr. Half of the thymocytes from each of these plates were used for FACS analysis under the conditions described by Telford et al. (1994), and the rest were used for DNA extraction.

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Cell Cycle Checkpoints: Preventing an Identity Crisis

Stephen J. Elledge

Cell cycle checkpoints are regulatory pathways that control the order and timing of cell cycle transitions and ensure that critical events such as DNA replication and chromosome segregation are completed with high fidelity. In addition, checkpoints respond to damage by arresting the cell cycle to provide time for repair and by inducing transcription of genes that facilitate repair. Checkpoint loss results in genomic instability and has been implicated in the evolution of normal cells into cancer cells. Recent advances have revealed signal transduction pathways that transmit checkpoint signals in response to DNA damage, replication blocks, and spindle damage. Checkpoint pathways have components shared among all eukaryotes, underscoring the conservation of cell cycle regulatory machinery.

The cell cycle is a collection of highly ordered processes that result in the duplication of a cell. As cells progress through the cell cycle, they undergo several discrete transitions. A cell cycle transition is a unidirectional change of state in which a cell that was performing one set of processes shifts its activity to perform a different set of processes. A current focus of cell cycle research concerns how these transitions are coordinated to occur at a precise time and in a defined order. In principle, the ordering of cell cycle events could be accomplished by requiring the next event to physically require the completion of the previous event, much like building a house—the roof cannot go up until the walls are built. This has been referred to as a substrate-product relationship (1). Alternatively, dependency could be established by positive or negative regulatory circuits, and this appears to be the predominant mechanism. An example of a pathway of cell cycle events that is subject to positive and negative

control is shown in Fig. 1A. A negative circuit is shown leading from b to a step in the d to e pathway. A positive circuit, shown linking events b and c, cannot be easily distinguished from a substrate-product relationship and depends upon the biochemical function of the step in question. These regulatory circuits are surveillance mechanisms that monitor the completion of critical cell cycle events and allow subsequent cell cycle transitions to occur. There are two classes of regulatory circuits, termed here intrinsic and extrinsic. Intrinsic mechanisms act in each cell cycle to order events. Extrinsic mechanisms are induced to act only when a defect is detected. Both mechanisms may use the same components to enforce cell cycle arrest. An example of how some of these circuits are integrated into a typical cell cycle is shown in Fig. 1B. These pathways are of considerable interest because their loss leads to reduced fidelity of cell cycle events such as chromosome duplication and segregation. Such alterations decrease the reproductive fitness of unicellular organisms and in multicellular organisms may lead to uncontrolled proliferation and cancer.

Checkpoint is the name given to a par-

ticular subset of these intrinsic or extrinsic mechanisms (1). A checkpoint is a biochemical pathway that ensures dependence of one process upon another process that is otherwise biochemically unrelated. A null allele in a checkpoint gene results in a loss of this dependency and, thus, checkpoints are inhibitory pathways. This definition of a checkpoint is broad and can apply to many situations that occur in multicellular organisms, particularly during development. However, its most common usage is in reference to control of cell cycle transitions. The word checkpoint conjures visions of both a place (a border) and a process (examination) and this duality has led to some confusion. The word is often used in a manner suggesting that checkpoints are points in the cell cycle or are cell cycle transitions, but the usage is best restricted to refer to the biochemical pathway that ensures dependency. For example, the DNA-damage checkpoint is the mechanism that detects damaged DNA and generates a signal that arrests cells in the G1 phase of the cell cycle, slows down S phase (DNA synthesis), arrests cells in the G2 phase, and induces the transcription of repair genes. The position of arrest within the cell cycle varies depending upon the phase in which the damage is sensed. Whether the loss of a checkpoint has an immediate consequence for an organism during a normal cycle depends on the particular pathway and the inherent timing of the processes themselves. Timing and checkpoints can act as redundant controls to ensure the proper order of events. Thus, there are no constraints on whether checkpoints are essential or inessential (extrinsic).

The first indications that the cell cycle was not controlled strictly by a substrate-product relationship came from cell fusion experiments in *Physarum polycephalum* that showed that timing of mitotic entry could be influenced by the ratio of the nuclear volume to cytoplasmic volume (2). Similar experiments with mammalian cells showed that when cells in S and G2 phases of the cycle were fused, the G2 nucleus delayed mitotic entry until the S-phase nucleus finished DNA replication; then both nuclei synchronously entered mitosis (3). This was interpreted to mean that S-phase nuclei produced an inhibitor of mitosis. The first example of a dependency relationship relieved by mutation was from bacterial studies. DNA damage and certain mutations cause a block to septation resulting in filamentation of *Escherichia coli* (4), and mutations in the *recA*, *lexA* and *sulA*(*sfIA*) genes relieve this septation block (5). SulA is an inhibitor of septation induced in response to DNA damage as part of the SOS response (6) controlled by *recA* and the repressor *lexA*. In eukaryotes, cells from hu-

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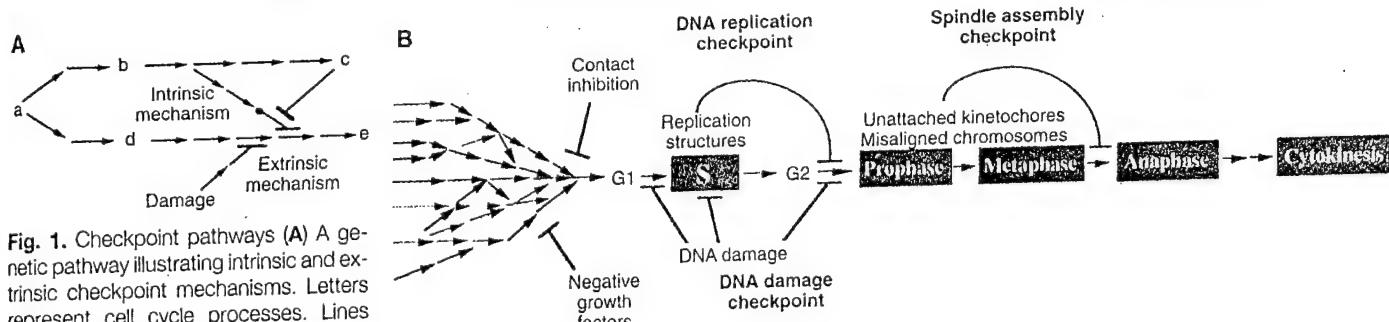


Fig. 1. Checkpoint pathways (A) A genetic pathway illustrating intrinsic and extrinsic checkpoint mechanisms. Letters represent cell cycle processes. Lines with arrowheads represent positively acting steps. Lines with perpendicular ends represent inhibitory steps. A dot at the beginning of a symbol indicates that once activated, it maintains its function without the need for upstream signals. The pathway shown as red symbols indicates an intrinsic checkpoint mechanism that operates to ensure that event C is completed before event E. After event B is completed, an inhibitory signal is activated that blocks completion of event E. After event C is completed, a signal is sent to turn off the inhibitory signal from B, thereby allowing completion of E. The blue symbols represent an extrinsic mechanism that is activated when defects such as DNA damage or spindle errors are detected. It is arbitrarily located on the D to E pathway but could also function by inhibiting a later step in the B

to C pathway. In that case, the extrinsic pathway would utilize the intrinsic mechanism for cell cycle arrest. Mutations in any of the red or blue symbols would result in a checkpoint-effective phenotype. (B) Schematic representation of several cell cycle checkpoints. The colored arrows depict complex signaling pathways that operate in G1 to transmit information regarding cell proliferation. The red lines connecting particular events and cell cycle transitions represent the inhibitory signals generated by checkpoint pathways in response to those events. The points of contact of the negative growth factor and contact inhibition pathways with the cell cycle are arbitrary and meant to indicate arrest in G1.

manns with the recessive disorder ataxia telangiectasia (AT) fail to show the reduction in rate of DNA synthesis and mitotic delay in response to DNA damage characteristic of normal cells (7). This was interpreted to mean that these cells were defective in the ability to coordinate cell cycle transitions in response to DNA damage, an interpretation that stands today. However, it was not until studies in the yeast *Saccharomyces cerevisiae* revealed the effects of the *rad9* mutation on cell cycle progression in response to DNA damage that the significance of the earlier studies began to be fully realized for eukaryotes (8). The identification of checkpoints in a genetically tractable organism known for its cell cycle genetics facilitated the generalization of these concepts to other aspects of the cell cycle and provided a basis for understanding much of its higher-order regulation. Furthermore, the connection between checkpoint failure, DNA damage sensitivity, and genomic instability in AT and *rad9* mutants provided an important insight into processes contributing to cellular dysfunction in cancer (9).

The identity of cell cycle phases is established not only by what they are in terms of the genes that are expressed and the processes that are executed, but also by what they are not. Cells in one phase often actively inhibit the processes of other phases through checkpoints (3). One notion concerning the origin of these inhibitory pathways derives from ideas about how cells evolved distinct S and M phases. Primitive cells may have initially performed S and M phases simultaneously like bacteria, but then evolved into simple oscillators that alternated between DNA replication and mitosis as genomes grew more complex. As

these two states became biochemically incompatible, strong selective pressure would have existed for a mechanism to inhibit the function of the previous state during a transition. If an inhibitory mechanism persists until the next change of state, it provides an inhibitory barrier that must be overcome, a checkpoint. Such molecular logic whereby a transition turns off the previous state while promoting the future state is a hallmark of cell cycle transitions (10, 11).

Cdk as Key Regulators of Cell Cycle Transitions and Effectors of Checkpoints

Several cell cycle transitions are dependent upon the activity of cyclin-dependent kinases (Cdks), and inhibition of these kinases is a mechanism by which some checkpoint pathways cause cell cycle arrest. These enzymes are composed of a kinase

subunit, Cdk, and an activating subunit, cyclin, in a complex that is subject to many levels of regulation (Fig. 2) (12). There appears to be a single cell cycle Cdk in *S. cerevisiae*, Cdc28, and *Schizosaccharomyces pombe*, Cdc2, whereas mammals have several—Cdc2, Cdk2, Cdk3, Cdk4, and Cdk6—that are specialized for different transitions. Cyclins are absolutely required for kinase activity and also contribute to substrate specificity. The Cdk-cyclin complex is both positively and negatively regulated by phosphorylation. In addition, Cdks are further regulated by binding to inhibitors (CKIs) and other proteins such as Suc1 (Cks1) that might modify their specificity or accessibility to regulators (13). Each of the proteins that influence Cdk activity are potential interfaces for signal transduction pathways that regulate cell cycle transitions and thus are potential targets for manipulation by checkpoint pathways.

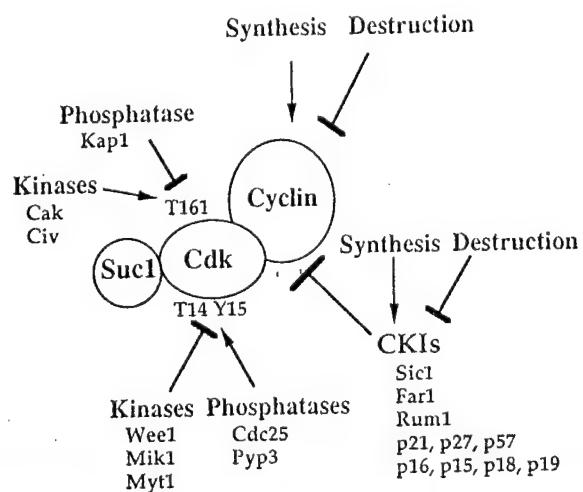


Fig. 2. Regulation of cyclin-dependent kinases. Arrowheads represent activating events and perpendicular ends represent inhibitory events. Genes known to perform the indicated functions are listed below. Both cyclins and some CKIs (Cdk inhibitors) are regulated by synthesis and ubiquitin-mediated proteolysis. Checkpoint pathways could act to promote inhibitory pathways or inhibit activating pathways to cause cell cycle arrest.

DNA Damage and DNA Replication Checkpoints in *S. cerevisiae*

Many checkpoint pathways have been identified primarily through the analysis of *cdc* (cell division cycle) mutants in yeast. Among these are checkpoints that sense mating partners, coordinate cell size and cell cycle progression, inhibit mitosis while in G1, make nuclear division dependent upon budding, restrict DNA replication to once per cell cycle, and make DNA synthesis dependent upon G1 cyclins. In this re-

view, I focus on the best defined pathways, the DNA damage and DNA replication checkpoints and the spindle-assembly checkpoint (Fig. 1B).

In response to DNA damage and blocks of DNA replication, cells from both prokaryotes and eukaryotes induce a set of physiological responses thought to facilitate DNA repair processes. Among these responses are cell cycle arrest in G1, S phase, and G2, a slowing of DNA replication, and increased transcription of genes encoding proteins that participate in DNA replication and repair (1). In some organisms, an

additional response, apoptosis, exists but is not explored in this review. Checkpoint-dependent arrest is thought to prevent the replication of damaged templates and the segregation of broken chromosomes. Since checkpoints are signal transduction pathways, they will be discussed in terms of their initiating signals, sensors, transducers, and effectors. A current view of the genetic organization of these pathways in *S. cerevisiae*, *S. pombe*, and mammals is shown in Fig. 3. The most striking feature of these pathways is that they share at least one common component in the signal transduction branch of the pathway, a phosphoinositide (PI) kinase superfamily member, indicating evolutionary conservation. Secondly, in the yeast pathways, the same signal transduction conduit is used both for the DNA damage checkpoint and arrest in response to replication blocks. I will use the budding yeast (*S. cerevisiae*) pathway as the primary example and discuss fission yeast and mammals when there are important differences.

DNA damage sensors and signal modifiers. Once DNA damage occurs, it can be processed through various repair pathways. These modifications may be required to produce the actual checkpoint signal. In

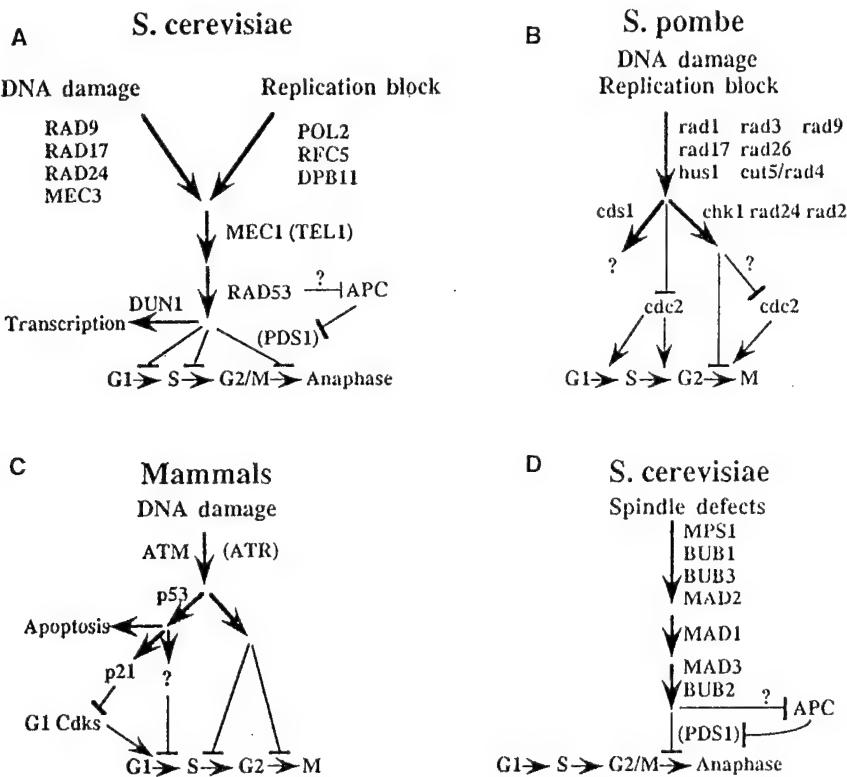


Fig. 3. A current view of the genetic organization of checkpoint pathways. The order of function of genes in groups along a single arrow is unknown and the order listed is arbitrary. (A) The DNA damage and DNA replication checkpoint in *S. cerevisiae*. *RFC5* and *DPB11* have not been examined for their G1 and G2 checkpoints and are therefore tentatively placed with *POL2*. *PDS1* has not been ordered genetically relative to *MEC1* and *RAD53* but is tentatively placed in parentheses at the end of the pathway because it alone is involved in both the DNA damage and the spindle checkpoints. The inhibitory connection between *RAD53* and the APC is hypothetical and is meant to indicate that *PDS1* might be regulated indirectly through regulation of the APC. Thus, this branch is an alternative to the inhibitory connection between *RAD53* and the G2/M to anaphase transition arrow. *TEL1* is in parentheses to indicate a minor redundant role with *MEC1*. G2/M is meant to indicate that arrest prior to anaphase can be considered either G2 or metaphase. (B) The DNA damage and DNA replication checkpoint in *S. pombe*. Arrows leading away from *cds1* indicates its function in an as yet undefined checkpoint function. The inhibitory circuit between *chk1* and *cdc2* is hypothetical and is based upon the putative role of tyrosine phosphorylation in the DNA damage checkpoint. It is meant as a possible alternative to the direct inhibition shown, not as a redundant pathway. The branch affecting the G1/S transition is assumed to exist but has not been demonstrated experimentally. Although the order of gene function shown is accurate, it is not clear that replication blocks and damage activate the pathway to equivalent degrees because modification of *chk1* is not observed in response to replication blocks (53). (C) The DNA damage checkpoint in mammals. ATM is shown transducing a signal to activate p53 which in turn activates the Cdk inhibitor p21 and a second unknown pathway. Little is known about the genes involved in the S phase slowdown and G2 arrest. (D) The *S. cerevisiae* spindle assembly checkpoint. *PDS1* is tentatively placed at the bottom of the pathway but has not been ordered genetically relative to the other genes shown.

Table 1. Homologs of DNA replication and damage checkpoint genes in yeast and humans. Genes are aligned on the basis of structural as opposed to functional similarities. Assumed biochemical activities are based on sequence similarity in some cases. Genes marked with an asterisk have not yet been shown to have checkpoint defects. Abbreviations: RFC, replication factor C; PIK, phosphoinositide kinase; PK, protein kinase; TF, transcription factor; CKI, cyclin-kinase inhibitor. Dashes indicate that a homolog has not yet been identified. In the case of *S. cerevisiae* which is completely sequenced, dashes indicate that highly related sequences have not been detected in the database. In the case of 14-3-3 proteins in humans, at least 7 genes have been identified. Of the *S. cerevisiae* genes listed only *CHK1* and *BMH1* and 2 have not yet been shown to have checkpoint function.

<i>S. cerevisiae</i>	Activity	<i>S. pombe</i>	Human
<i>RAD9</i>		—	—
<i>RAD24</i>	RFC-related	<i>rad17</i>	—
<i>RAD17</i>	Nuclease	<i>rad1</i>	—
<i>MEC3</i>		—	—
<i>MEC1</i>	PIK	<i>rad3</i>	ATR*
<i>TEL1</i>	PIK	—	ATM
<i>RAD53</i>	PK	<i>cds1</i>	—
<i>POL2</i>	Polymerase	<i>cdc30*</i>	<i>Pol ε*</i>
<i>DPB11</i>		<i>cut5</i>	—
<i>CHK1*</i>	PK	<i>chk1</i>	—
<i>PDS1</i>		—	—
<i>DUN1</i>	PK	—	—
<i>BMH1,2*</i>	14-3-3	<i>rad24, 25</i>	14-3-3*
—		<i>rad26</i>	—
—		<i>rad9</i>	<i>HRAD9*</i>
—	TF	—	<i>p53</i>
—	CKI	—	<i>p21</i>

E. coli, there is considerable evidence to suggest that some types of DNA damage are converted to single-stranded DNA (ssDNA) that is then bound by RecA; it is this recombination complex that is sensed by LexA. In *S. cerevisiae*, it is not clear whether there are several sensors that can detect different types of damage, whether all damage is processed to one or a few forms that can be sensed by a limited number of sensors, or whether it is the actual process of repair that is sensed. We know that the presence of ssDNA correlates with arrest through analysis of *CDC13*. *CDC13* encodes a protein that binds to telomeres and protects them from degradation (18). Temperature-sensitive *cdc13* mutants accumulate ssDNA at the telomeres and arrest in the G2 phase of the cell cycle (14). Four genes, *RAD9*, *RAD17*, *RAD24*, and *MEC3* have properties consistent with a role as signal modifiers or sensors (15, 16). They are required for G2 arrest of *cdc13* mutants and also for arrest in G1 and G2 in response to DNA damage, but not in response to a blockade of replication. Interestingly, *rad17*, *rad24*, and *mec3* mutants decrease the amount of ssDNA that accumulates in *cdc13* mutants, whereas *rad9* mutants increase the amount of ssDNA accumulation. Furthermore, *rad17* is structurally related to the *U. maydis* checkpoint gene *rec1*, a 3'-5' exonuclease (16, 17), suggesting that *rad17* is involved in modifying damage to generate a signal. (However, if *cdc13* mutants degrade their DNA from the telomere, they would use a 5'-3' exonuclease to generate the single strand observed in vivo, not a 3'-5' exonuclease.) *Rad24* and its *S. pombe* counterpart *Rad17* are related to *RFC*, a protein that binds gapped DNA. Thus, they are implicated in damage recognition although no biochemical function has been demonstrated (19). Because ssDNA is present in *cdc13rad9* mutants but fails to cause arrest, *Rad9* must be a sensor or transducer of the DNA damage signal.

DNA replication sensors and signal transducers. Structures in the replication complex or unreplicated DNA may send signals to inhibit mitotic entry (3). A persuasive argument against signaling by unreplicated DNA is that several mutants that block the initiation of DNA replication, such as deletions of *Pol α* (20), *cut5* (21), or *cdc18* (11) in *S. pombe* and *CDC6* in *S. cerevisiae* (22) allow mitosis to proceed with unreplicated DNA. However, it is a formal possibility that the unreplicated DNA in these mutants is in a different "non-signaling state" than the unreplicated DNA in cells whose cycles have already progressed into S phase. Furthermore, it is not certain whether the replication checkpoint is active constitutively once DNA replication has initiated,

or merely becomes activated when replication is blocked. There is an inducible transcriptional component to the pathway that is not constitutively active during S phase (23), suggesting that the arrest mechanism is also inducible. So far none of the mutants defective for the checkpoint pathway allow mitosis to occur sooner than it normally does in an unperturbed cycle, but this may reflect the inherent timing of DNA replication and mitosis.

Three DNA replication genes required for the DNA replication checkpoint in *S. cerevisiae*, *POL2* (24), *DPB11* (25), and *RFC5* (26) are candidate sensors of DNA replication. *POL2* encodes DNA polymerase ε (Pol ε), which is required for chromosomal DNA replication (27). The *pol2* checkpoint-defective mutants are proficient for G1 and G2 arrest in response to DNA damage, but are defective in the transcriptional response and the ability to prevent mitotic entry when replication is blocked by hydroxyurea (HU), an inhibitor of ribonucleotide reductase. *DPB11* is the *S. cerevisiae* homolog of *S. pombe* *cut5*, which is required for DNA replication and for arrest in response to HU (28). *RFC5* is a component of replication factor C that binds to gapped DNA, such as that present on the lagging strand during replication, and recruits proliferating cell nuclear antigen (PCNA), which in turn recruits DNA Pol δ and Pol ε. A polymerase could function as a sensor of DNA replication because it is located at the replication fork. However, at the current level of resolution, it is impossible to distinguish between a sensory role versus a signal transduction role for any of these proteins and it is possible that it is the activity of an entire complex that must be intact to properly sense replication.

POL2 and *RAD9* (and *RAD17*, *RAD24*, *MEC3*) participate in temporally alternative branches of the pathway for sensing DNA damage. DNA damage incurred during S phase is sensed largely in a *POL2*-dependent manner, whereas damage incurred in G1 and G2 is primarily dependent upon *RAD9* (29), and *pol2rad9* double mutants are completely defective for the transcriptional response in all phases of the cycle. This is consistent with their complementary roles in activating cell cycle arrest. However, *rad9* mutants do show reduced slowing of DNA replication in response to the methylating agent methyl methane sulfonate (30) and, although *rad9* mutants alone are not HU-sensitive, they greatly enhance the HU-sensitivity of *pol2* mutants. This suggests a minor or redundant role for *RAD9* in the S-phase checkpoint pathway.

Signal transducers. Two essential genes form the central conduit for checkpoint signal transduction in *S. cerevisiae*, *MEC1*

(*ESR1*, *SAD3*) (15, 23, 31) and *RAD53* (*SPK1*, *MEC2*, *SAD1*) (15, 23, 32). All cell cycle arrest, reduction in the rate of DNA replication, and transcriptional responses to DNA damage and incomplete replication are dependent upon these two genes (23, 29, 33). *MEC1* is a member of the PI kinase superfamily, some members of which are protein kinases. The *Mec1* homolog in *S. pombe*, *Rad3*, has an associated protein kinase activity that is dependent upon a functional kinase domain within *Rad3* (34). While short of formal proof, these results strongly suggest that *Rad3* and *Mec1* are protein kinases. *TEL1*, which is required for telomere length maintenance (35), is a structural homolog of *MEC1* (36, 37). Although *tel1* mutants have functional checkpoints, mutations in *TEL1* enhance the sensitivity of *mec1* mutants to DNA damage and therefore *TEL1* has a minor checkpoint role.

To date, *MEC1* (*rad3*) and *TEL1* are the only checkpoint genes conserved in a functional sense among higher eukaryotes (see Table 1). Homologs include *mei-41* (32) in *Drosophila melanogaster*, and *ATM* (ataxia telangiectasia mutated, 39) and *ATR* (AT and *rad*-related, 34) in mammals. *ATM* mutant cells have defective G1 and G2 DNA damage checkpoints and show radiation-resistant DNA synthesis, for example, they do not slow replication in response to damage, and therefore share a subset of the *mec1* and *rad3* phenotypes (7, 40). *ATM* is more closely related to *TEL1*, whereas *ATR*, also known as *FRP* (FRAP-related) (41), *MEC1*, and *rad3* form a separate subfamily. Although the function of *ATR* is unknown, *ATR* and *ATM* bind to distinct and complementary portions of *mei-41* chromosomes suggesting a possible role in signaling different stages of meiotic progression or perhaps in the recombination process itself (43). Mutants in *mec1* (*esr1*) have been reported to be defective in meiotic recombination (31, but see 42) and in cell cycle arrest when recombination is blocked (42).

RAD53 is a protein kinase that is phosphorylated and activated in response to DNA damage. Phosphorylation of *Rad53* is dependent upon *POL2*, *RAD9*, and *MEC1* (29, 37, 44). This and other data (37) indicate that *RAD53* functions downstream of *MEC1*, *POL2*, and *RAD9* to transduce the signal from DNA damage and incomplete replication and may be a substrate of *Mec1*. Modification of *Rad53* in response to DNA damaging agents is much more pronounced than that achieved in response to HU, indicating complexity in the upstream signaling process (37). Although checkpoint defective, *rad53* mutants are much less sensitive to UV and HU than *mec1* mutants, indicating that *Mec1* controls processes that are not solely dependent upon *Rad53*.

Effectors of the transcriptional response. In parallel to the cell cycle arrest response to DNA damage and replication blocks is a separate transcriptional response specifically controlled by the protein kinase Dun1 (29, 45). Although there is currently no evidence that the transcriptional response is involved in cell cycle arrest in *S. cerevisiae*, it is clearly involved in arrest in mammals and for this reason is included here. Dun1 kinase activity is increased by DNA damage in a RAD53- and MEC1-dependent manner, and this activation is required for transcriptional activation of the genes encoding ribonucleotide reductase, *RNR1*, *RNR2*, and *RNR3*. However, *DUN1* does not appear to have a unique role in cell cycle arrest. Presumably there will be a series of transcription factors analogous to p53 that are altered in a Dun1-dependent fashion to activate transcription. Other mutants in this portion of the pathway include the *crt* (constitutive *RNR3* transcription) mutants (46), three of which—*SSN6*, *TUP1*, and *CRT1*—are epistatic to *dun1* and are therefore likely to function downstream of *DUN1* or in a separate pathway. Because *TUP1* and *SSN6* are general components of transcriptional repressors, part of the transcriptional response may be accomplished by alleviating repression.

Effectors of the cell cycle arrest response. Organisms may differ in their requirements for blocking mitotic entry depending upon how their cell cycles are organized. *S. pombe*, *Xenopus laevis*, and mammals can effectively prevent the G2-to-metaphase transition by blocking Cdk activity. However, *S. cerevisiae* starts spindle assembly during S phase, effectively initiating mitosis, and in order to inhibit cell cycle progression they must block entry into anaphase. This is likely to be accomplished by a mechanism distinct from that used to block entry into metaphase because they are different biochemical steps (see the discussion of *cdc55* mutants in the spindle assembly checkpoint). The best candidate for an effector of cell cycle arrest in response to DNA damage is *PDS1*, an anaphase inhibitor. The *pds1* mutants fail to arrest in G2 in response to gamma irradiation (γ -IR) or in the presence of *cdc13* mutations (47). Furthermore, *pds1* mutants are also defective in the spindle assembly checkpoint, indicating a potentially common target for these two checkpoint pathways. *Pds1* is degraded by ubiquitin-mediated proteolysis by a set of proteins that promote anaphase, the anaphase-promoting complex (APC). *PDS1* mutants resistant to destruction cause a pre-anaphase arrest (48), so blocking *Pds1* destruction is one mechanism by which cells could respond to DNA damage to prevent mitosis and allow

time for repair. Whether *Pds1* is directly modified in response to DNA damage, or whether it is indirectly regulated through control of the APC remains to be determined. Because failure to degrade mitotic cyclins causes arrest after anaphase, it is unlikely that arrest in response to DNA damage is mediated by complete inhibition of APC function. Indeed, γ -IR of *pds1* mutants allows progression through telophase into the next cycle. In addition, *pds1* mutants do arrest if DNA replication is blocked, indicating the existence of a distinct S phase-specific effector. *Pds1* could have an indirect role in checkpoint function. For example, *Pds1* may be required for prolonged cohesion of newly replicated sisters during checkpoint arrest. If the process of sister separation, once begun, sends a positive signal for progression through the cell cycle, *pds1* mutants could simply bypass the checkpoint signal by initiating anaphase.

The *S. pombe* Checkpoint Pathway

The organization of the *S. pombe* checkpoint pathway (Fig. 3B) is similar to that of the *S. cerevisiae* pathway, and these pathways share several conserved genes (Table 1). A group of 7 genes, *rad1*, *rad3*, *rad9*, *rad17*, *rad26*, *hus1*, and *cut5* are required for cell cycle arrest in response to both damage and replication blocks (49). The protein kinase *cds1* is very similar in sequence with the first 70% of the *S. cerevisiae* Rad53 protein, is required for survival during cell cycle arrest with HU (50). Cell cycle arrest by DNA damage but not blocked replication requires the function of the Chk1 (Rad27) protein kinase (51) and the 14-3-3 proteins encoded by *rad24* and *rad25* (52). Chk1 becomes phosphorylated in response to DNA damage in a Chk1-dependent manner (53). This presumed autophosphorylation is dependent upon the *rad1*, *rad3*, *rad9*, *rad17*, *rad24*, and *hus1* genes and *chk1* is therefore placed downstream of these genes. Major similarities between the yeasts include structural similarity between checkpoint genes: *MEC1* and *rad3* (34, 54), *RAD17* and *rad1* (16), *RAD53* and *cds1* (50), *RAD24* and *rad17* (19), and *DPB11* and *cut5* (25). Furthermore, signals from both DNA damage and blocked replication are transduced through a common pathway in both organisms.

There are also significant differences in checkpoint control: (i) unlike *MEC1* and *RAD53*, *rad3* and *cds1* are not essential, (ii) *rad53* mutants behave differently than *cds1* mutants when DNA replication is blocked, (iii) *S. pombe* *rad17* is required for both the damage and replication

checkpoints whereas *S. cerevisiae* *RAD24* is required only for arrest by DNA damage, (iv) cell cycle arrest in response to blocked replication (55) and possibly damage (56) requires inhibitory phosphorylation of a tyrosine on *S. pombe* Cdc2 but this is not the case in *S. cerevisiae* (49).

Without knowledge of the essential roles of *RAD53* and *MEC1*, it is difficult to explain why the two yeasts differ in this respect. One explanation may be that the timing of mitosis relative to the end of DNA synthesis differs in the two yeasts. Unlike *S. cerevisiae* which exerts its size control in G1, *S. pombe* integrates its size control primarily during G2 to provide an additional, perhaps redundant, delay before mitotic entry. Support for this hypothesis is that *wee1-50* mutants, which accelerate mitosis to produce smaller *S. pombe* cells, are lethal in combination with mutants of the *rad3* group or *chk1* (51, 57). However, an alternative explanation that cannot be eliminated is that *wee1-50* mutants cause a DNA replication problem that requires *rad3* function.

The fact that the *S. pombe* *rad17* mutant is defective for both the damage and replication checkpoints while *S. cerevisiae* *RAD24* is required only for arrest by DNA damage may mean that they are not true homologs, although they are structurally related. Alternatively, this discrepancy may underscore differences in how replication blocks are sensed in the two organisms.

Unlike *rad53* mutants, *cds1* mutants do not enter mitosis in the presence of HU (50). However, once HU is removed, *cds1* mutants complete the bulk of DNA synthesis and undergo a mitotic catastrophe in which chromosomes fail to properly segregate prior to septation. While the difference in cell cycle arrest remains unclear, the delayed mitotic catastrophe is potentially revealing and raises the possibility that the response to DNA replication blocks may include essential functions other than simply preventing mitosis. Support for this also comes from the fact that the loss of viability of *rad53* mutants in HU cannot be suppressed by blocking mitosis with microtubule inhibitors (23). One such additional function might be controlling the integrity of stalled replication complexes. A partial loss of replication fork integrity could result in the disassembly of replication forks. While loss of a few forks would not necessarily be a catastrophic event, if two converging forks collapse (CFC), the intervening DNA is not replicated. Failure to prevent or repair CFC will lead to segregation of partially replicated chromosomes and catastrophe.

In *S. pombe*, tyrosine phosphorylation of Cdc2 is necessary for proper arrest in response to blocked replication (55). It is not

known whether this phosphorylation is directly regulated by the checkpoint pathway or merely required for proper checkpoint function. Recent experiments concerning the replication checkpoint in *X. laevis* extracts indicate that tyrosine phosphorylation alone cannot explain the observed cell cycle arrest. An undefined Cdk inhibitor activated by replication blocks was detected (58). Experiments in *Aspergillus nidulans* have implicated redundancy between Cdc2 tyrosine phosphorylation and a *bimE*-dependent pathway (59). The *bimE* protein is a component of the APC and *bimE* mutants are likely to have unusually large amounts of cyclin B. Tyrosine phosphorylation is required for arrest in response to DNA damage in *A. nidulans* (60). Because the proteins that control tyrosine phosphorylation are known, it is clear that the next step is to determine how (and if) DNA damage and interference with DNA replication affects the activities of these enzymes and to what extent the APC is involved in arrest.

Mammalian DNA Damage Checkpoints

Mammals have the same cell cycle responses to DNA damage as yeast, but in addition may activate a cell death pathway. Cell elimination is a viable strategy for metazoans because their goal is not the survival of each damaged cell, which might harbor mutations, but the survival of the organism. I will not discuss the apoptotic response. Of the various mammalian checkpoints, only the G1 DNA damage checkpoint is understood in any detail. Three mammalian genes control the DNA damage checkpoint, mutated in ataxia telangiectasia (ATM) (7, 39), p53 (61), and p21 (62). Of these, p53 is the most widely studied. The p53 gene is the tumor suppressor most frequently mutated in human cancers (63). It encodes a transcription factor that is activated in response to DNA damage and perturbation of nucleotide pools. Cells defective for p53 are unable to arrest in G1 in response to γ -irradiation and show reduced apoptosis. Part of p53's ability to arrest G1 cells results from activation of transcription of p21, a tight-binding inhibitor of Cdks that control entry into S phase (64). Mouse embryo fibroblasts lacking p21 show a partial defect in G1 arrest that is less severe than that of p53-defective fibroblasts, indicating that a second p53-dependent G1 arrest pathway exists. While the nature of this pathway is not known, experiments with mutant forms of Cdk4 have suggested that tyrosine phosphorylation of Cdk4 may be required for G1 arrest in response to UV irradiation (65) and is therefore a good candidate for the p21-independent path-

way. The p21 protein has also been shown to control checkpoint function in human cells (66). It is not known how yeast cells arrest in G1, but failure to destroy inhibitors such as Sic1 (or Far1 in the case of α -factor arrested cells) could provide a mechanism similar to inhibition by p21.

How p53 is activated in response to DNA damage is still unknown. Both its stability and specific activity as a transcription factor appear to increase in response to DNA damage, but the precise mechanism has remained elusive despite intensive study (63). The ATM gene has been implicated in regulation of p53. Cells lacking ATM show a reduced and delayed activation of p53 in response to DNA damage (67). Given its relationship to MEC1 and *rad3*, it is likely that ATM plays a role in transducing the DNA damage signal to p53. Although ATM is upstream of p53, ATM mutants die via p53-dependent apoptosis in response to DNA damage. Therefore, an ATM-independent mechanism for p53 activation must exist, perhaps controlled by ATR.

The fact that both p53 and ATM are frequently mutated in human cancers strongly implicates checkpoint function in the prevention of cancer. How much we can learn about cancer from the analysis of yeast checkpoints will depend upon the degree of conservation between the human and yeast pathways. So far, few human checkpoint genes have been identified and of these, only ATM has yeast homologs (Table 1). However, it appears that budding and fission yeasts share many conserved checkpoint genes, and it is often the case that genes shared among these organisms are common to all eukaryotes. With the rapid advances occurring in the identification of genes in various sequencing projects, a definitive answer to this question should be known in the very near future. In this regard, a human homolog of the *S. pombe* *rad9* gene has been identified, although its role in animal cells has yet to be determined (68). Furthermore, the genes that control cell cycle arrest in response to DNA damage also control other aspects of the DNA damage response, possibly specific DNA repair pathways and apoptosis. Therefore, although it is generally assumed that the loss of the ability to arrest the cell cycle leads to genomic instability and cancer, we are actually far from having definitively proven that fact. Proof will require specific elimination of the ability to arrest the cell cycle without affecting the rest of the signaling pathway. In the one case where this has been accomplished, loss of p21 in the mouse, cancer did not result (62), and p21 mutations are very rare in human tumors. Therefore, this question will require future scrutiny.

The Spindle Assembly Checkpoint

The proper segregation of chromosomes requires the execution of a number of processes during mitosis: a bipolar spindle must be assembled; chromosomes must attach to the spindles through the kinetochores, a protein structure that forms on the centromeres of chromosomes; kinetochores of sister chromatids must bind to spindle fibers attached to opposite poles; and properly attached chromosomes must arrive at the metaphase plate. The spindle assembly checkpoint prevents the onset of anaphase, the actual segregation of chromosomes, until these processes have been properly accomplished. Once these events take place, cells can execute anaphase and progress into the next cell cycle.

Spindle assembly signals and sensors. While it is premature to discuss the molecular identity of sensors, there is a lively debate as to the nature of the event being sensed. The assembly of spindles involves many different components any of which could (and may) be sensed. As noted in a recent review (69), the sensor could detect the amount of free tubulin, the function of the microtubule organizing center, the bipolarity of the spindle, the attachment of microtubules to the kinetochore, or the tension generated on the kinetochore by attachment to a bipolar spindle. Because many of these processes are interdependent, it is possible that defects in any one could result in the failure of a common event that could be the signal for all defects. The leading candidates for signals are lack of chromosome attachment to the spindle and the absence of tension generated on a chromosome attached to a bipolar spindle.

Support for the sensing of tension at the kinetochore comes from experiments in which chromosomes were micromanipulated with glass needles. By manipulating chromosomes of grasshopper spermatocytes in meiosis, it is possible to force both attachments of sister chromatids to the same pole. While normally unstable, such a mono-oriented chromosome pair can be made stable and will persist until anaphase if force toward the opposite pole is exerted on the chromosome by the needle (70). Furthermore, meiotic anaphase can be delayed by the presence of a chromosome lacking its synaptic mate. Applying force on that chromosome with a glass needle mimics tension from proper bipolar attachment to the spindle and causes rapid entry into anaphase (71).

Evidence supporting the sensing of unattached kinetochores derives from laser ablation of kinetochores in mammalian mitotic cells. If the unattached kinetochore of the last monoattached chromosome is destroyed by laser ablation, cells no longer delay an-

aphase entry even though there is a lack of tension on that chromosome (72). Additional evidence is that *S. cerevisiae* lacking *CDC6* and *S. pombe* lacking *cdc18* undergo mitosis with unreplicated chromosomes (11, 73). The absence of bipolar spindle attachment in this case should prevent tension but does not prevent anaphase. Furthermore, the absence of sisters precludes the need for the APC suggesting that the only role of ubiquitin-mediated proteolysis in anaphase is to allow sister separation (73). It is also possible that one mechanism operates in meiotic cells (tension) and a second in mitotic cells (attachment). Alternatively, a lack of tension may produce free microtubule-binding sites in the kinetochore and these may activate the checkpoint (69). This would accommodate both sets of observations. Regardless of the model, it is clear that the spindle assembly checkpoint is capable of detecting signals generated at the kinetochore. Genetic evidence supporting this is that mutants in genes encoding kinetochore proteins such as *Ctf13* or mutations in the centromere itself delay mitosis in budding yeast. Remarkably, higher eukaryotic cells can execute anaphase efficiently in the absence of kinetochores (74) or even chromosomes themselves (75)! This suggests that the presence of kinetochores establishes the checkpoint in the first place. It would be interesting to know whether spindle damage would prevent anaphase entry in the absence of chromosomes.

A molecular correlate has been identified for a signal regulated by tension. The monoclonal antibody 3F3 recognizes a phospho-epitope on an unknown kinetochore protein that appears much more abundantly on unattached kinetochores (76). The large amount of signal on a monoattached chromosome can be diminished by exerting a force toward the unattached pole to generate tension (77). Furthermore, injection of antibodies to 3F3 delays anaphase and the dephosphorylation of the 3F3 epitope, suggesting that this dephosphorylation may be necessary to turn off the checkpoint after all the chromosomes have been aligned on the spindle (78).

Signal transducers in the spindle assembly checkpoint. The genetic pathway responsible for the spindle assembly checkpoint is shown in Fig. 3D. The majority of these genes were identified in two screens for the failure to arrest in the presence of the microtubule depolymerizing drugs. *MAD1*, *MAD2*, and *MAD3* (mitotic arrest defective) (79) and *BUB1*, *BUB2*, and *BUB3* (budding uninhibited by benimidazole) (80) are not essential genes but their mutants attempt aberrant mitoses in the presence of microtubule inhibitors and die. The *mad* and *bub* mutants also show an increased frequency of sponta-

neous chromosome loss reflecting a role in detecting endogenous errors. These proteins are also required for delaying anaphase entry in the presence of chromosomes carrying mutant centromeres, indicating a role in detecting kinetochore-generated signals. *MPS1* was identified as a gene required for spindle pole body assembly and later was found to have a checkpoint phenotype (81).

The spindle assembly checkpoint signals through two protein kinases, *Mps1* and *Bub1* (82). Overproduction of *Mps1* arrests the cell cycle (83) and this arrest is dependent upon all of the *MAD/BUB* genes. This suggests that activation of *Mps1* may be one of the initial signaling events in the checkpoint pathway. *Mad1* becomes phosphorylated when the checkpoint is activated (84), and this event has been used to order the action of other genes in the pathway. *Mps1* directly phosphorylates *Mad1* in vitro and is required for its phosphorylation in vivo (83), indicating that this may be a critical signaling event. A complication exists in placing *Mps1* directly adjacent to *Mad1* in the signaling pathway because *Mad1* phosphorylation is also dependent upon *BUB1*, *BUB3*, and *MAD2* when the checkpoint is activated, and these genes are also required for cell cycle arrest when *Mps1* is overproduced. Clearly this is a complex signaling mechanism that cannot be easily organized using genetic analysis alone. *Mad2* is a protein that binds to *Mad1* and is required for its phosphorylation (69). *Mad2* may be a central protein in this signaling cascade because its localization to kinetochores changes under conditions that activate the signaling pathway. *Mad3* and *Bub2* are not required for *Mad1* phosphorylation and are therefore placed after *Mad1* in the signal transduction pathway.

The *Xenopus* homolog of *MAD2*, *XMAD2*, is required for the spindle assembly checkpoint in vitro (85) and the human homolog is required for checkpoint function in vivo (86). Both human and *Xenopus* *Mad2* localize to the kinetochores of unattached chromosomes. Once chromosomes attach to microtubules, *Mad2* immunostaining is lost. While possibly due to epitope masking, the more exciting possibility is that *Mad2* associates with unattached kinetochores and signals to activate the spindle checkpoint. Since in yeast *Mad2* binds to *Mad1* and is required for *Mad1* phosphorylation, it is possible that *Xenopus* *Mad1* is also localized to kinetochores and will have the phosphorylation properties attributed to the 3F3-reactive protein. A plausible model for how the spindle assembly checkpoint may operate is that in the absence of tension or presence of free microtubule binding sites, a protein kinase such as *Mps1* or *Bub1* is activated

and phosphorylates a protein localized at the kinetochore. This phosphorylation leads to recruitment of the *Mad2* protein which then connects the circuit allowing *Mad1* phosphorylation and generation of the arrest signal, perhaps through *Mad3* and *Bub2*. The binding of *Xmad2* and the model of sensing kinetochore attachment suggests that the spindle assembly checkpoint may not be inducible (extrinsic) in the same sense as the DNA damage pathway, but is active during each cell cycle when kinetochores mature. Microtubule inhibitors would prevent the proper assembly of the kinetochore microtubules and would thereby maintain the checkpoint signal.

Effectors of the spindle checkpoint pathway. Significant advances have recently been made concerning the mechanism of spindle checkpoint-dependent cell cycle arrest. Interference with ubiquitin-mediated proteolysis either by mutations in components of the APC in vivo or by inhibition with methyl ubiquitin in vitro can arrest cells before anaphase (R. W. King *et al.*, p. 1652), consistent with a role for proteolysis. Yeast arrested via this checkpoint have stable cyclins and high Cdk activity. Although cyclins are degraded by the APC, cyclins are not the critical substrate for the anaphase transition (87). As described above, degradation of the *Pds1* protein is dependent upon the APC and is required for anaphase entry. Furthermore, *pds1* mutants show checkpoint defects. Together, these observations provide evidence that *Pds1* is a potential effector of the spindle assembly checkpoint. The same caveats apply here as for the role of *Pds1* in DNA damage checkpoint. It remains to be determined precisely how *Pds1* degradation is controlled. Is the activation of the APC blocked by checkpoint activation, or is *Pds1* somehow protected from an activated APC?

A gene that is required for arrest by microtubule inhibitors but which may lie outside of the pathway shown is *CDC55*, a non-essential regulatory component of the PP2A phosphatase. Unlike *mad* and *bub* mutants which ignore the inhibitory signal of microtubule inhibitors, *cde55* mutants allow the separation of sister chromatids in the presence of nocodazole by inhibiting Cdc28 kinase activity through tyrosine phosphorylation (88). Thus, Cdc28 inactivation may be a secondary pathway allowing separation of sister chromatids. Whether this represents anaphase or a return to a premiotic state could not be determined because the experiments were done in the presence of nocodazole. Whether this pathway is used during arrest in wild-type cells remains to be determined. If so, it may represent an adaptation response in which unicellular organisms unable to repair a checkpoint

activating defect undertake a defective transition rather than remain terminally arrested. In this sense, an adaptation pathway is a method cells use for measuring time. Such an adaptation response has been suggested for DNA damage (89).

If the DNA damage and spindle assembly checkpoint arrest at the same position in the cell cycle as suggested by their dependency on the same effector, PDS1, this may explain why the DNA damage and replication checkpoints have evolved an arrest mechanism distinct from tyrosine phosphorylation in budding yeast. As noted for *cdc55* mutants, tyrosine phosphorylation of Cdc28 at that stage of the cell cycle may activate anaphase as opposed to preventing it.

The flexibility of checkpoint pathways. Once cell cycle arrest mechanisms are established it is possible that many signaling pathways can interface with a central pathway to utilize the same arrest mechanism. For example, the spindle assembly checkpoint in *X. laevis* requires the activity of a mitogen-activated protein (MAP) kinase, p44^{ERK2} (90). The arrest of mature oocytes in the second meiotic metaphase by CSF (cytostatic factor) also requires p44^{ERK2} and is likely to use the same arrest mechanism. Size control and DNA replication both require tyrosine phosphorylation in *S. pombe*. The DNA damage and replication checkpoints appear to use the same pathway in *S. cerevisiae*, *S. pombe*, and *A. nidulans*. *Drosophila melanogaster* uses tyrosine phosphorylation of Cdk5 to regulate cell cycle progression during development (49). These signal transduction pathways appear to be flexible modules that can be adapted to meet diverse evolutionary demands.

Summary

The last 8 years have seen a rapid increase in our knowledge of the regulation of cell cycle transitions. Many of the main cell cycle checkpoints have been identified and biochemical analysis of their signal transduction mechanisms are under way. For those that directly regulate Cdk activity, we have sufficient basic knowledge of Cdk regulation to uncover the mode of regulation. It is now a matter of connecting the signal transduction proteins to each other and to the direct effectors of Cdk function. For those pathways that operate through non-Cdk regulation such as PDS1, there is much to learn about how they carry out their inhibitory functions. Furthermore, we know very little about the mechanisms these pathways use to monitor cell cycle events.

Important questions remain as to the nature of checkpoints in mammals and the integration of checkpoint pathways with

cell proliferation controls and development. Inappropriate expression of the proto-oncogene cMyc can activate the p53-dependent checkpoint pathway (91). Do growth promoting pathways generally become integrated into checkpoint pathways as a consequence of cellular differentiation? Is this a mechanism of cancer prevention? Furthermore, what are the relative contributions of DNA repair, cell cycle arrest, and apoptosis to cancer prevention by checkpoint pathways? Are other checkpoints such as the spindle assembly pathway disrupted in tumors? Checkpoints figure prominently in therapeutic strategies to eliminate cancer cells. Most agents kill cancer cells by activating checkpoint-mediated apoptosis pathways or by exploiting chemical sensitivities due to loss of checkpoint function (9). In the future we should be able to exploit our increased understanding of checkpoints to further this cause. While we have learned much, we have only dipped beneath the surface of what we must know to fully understand checkpoints. Fortunately we now have the biochemical and genetic tools needed to address many of these interesting and important questions. These are stimulating times. So much so that it is virtually impossible to keep one's excitement in check, and that's the point.

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[17] Purification and Analysis of CIP/KIP Proteins

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Introduction

This chapter describes methods and reagents for preparation and analysis of cyclin-dependent kinase (Cdk) inhibitors of the CIP/KIP family. These inhibitors, which include mammalian p21^{Cip1,1-3}, p27^{Kip1,4-5}, and p57^{Kip2,6-7} and the *Xenopus* homologs p28^{Xic1,8-9} and p27^{Xic1,8-9} are tight binding inhibitors of cyclin/Cdk complexes, with K_i values typically in the nanomolar range.¹⁰ Association of CIP/KIP proteins with Cdks is dependent on the presence of a cyclin subunit^{10,11} and the X-ray structure of the p27/Cdk2/cyclin A structure has revealed that strong inhibitor contacts are made with both the cyclin and the Cdk subunits.¹² This article describes (1) structural and functional domains of CIP/KIP proteins, (2) expression systems for CIP/KIP proteins, (3) methods for assaying recombinant CIP/KIP proteins, and (4) production and characterization of monoclonal antibodies against p21 and p57. Other aspects of CIP/KIP protein function are reviewed elsewhere.¹³⁻¹⁶

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Domain Structure

Based on structural and biochemical characterization, the CIP/KIP family members contain minimally three domains (Fig. 1).^{4,10} The N-terminal ~60 amino acid fragment of CIP/KIP proteins contains both a cyclin-binding domain and a Cdk-binding domain, and is sufficient for kinase inhibition. The residues involved in inhibition have been deduced through an analysis of the crystal structure of the cyclin A/Cdk2 bound to a 69 amino acid inhibitory fragment of p27.¹² This structure reveals that p27 associates with both the cyclin and Cdk proteins, the latter interaction involving large structural changes in the Cdk2 N-terminal lobe. The LFG sequence in p27 (residues 32–34) binds to a shallow groove on the surface of cyclin A generated by a highly conserved region of the cyclin box (MRAILVDW, underlined residues are highly conserved). This region of p27 is among the most highly conserved regions in the CIP/KIP family. Interestingly, this LFG motif is found in p107, a tight-binding Cdk2/cyclin

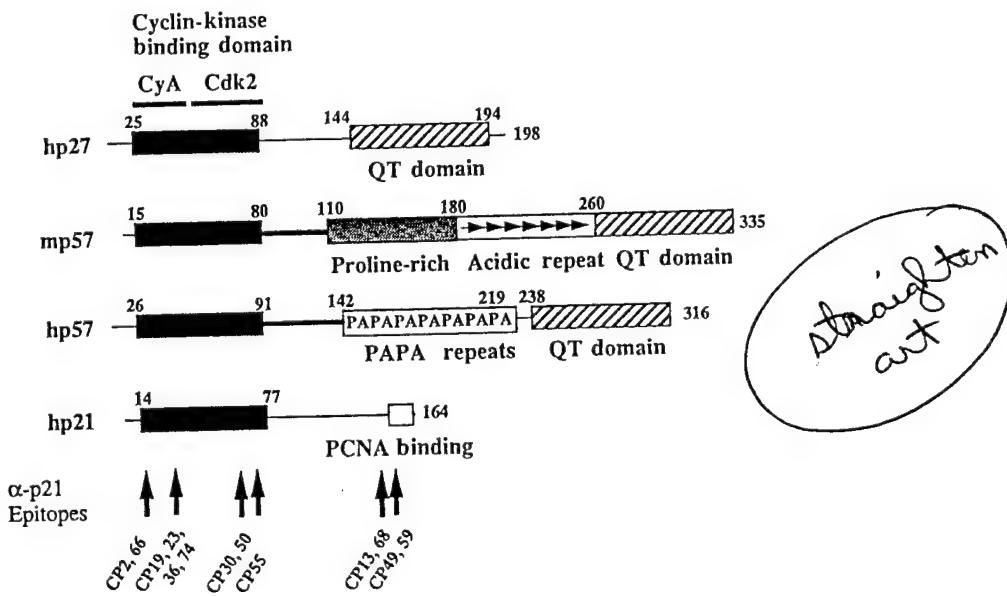


FIG. 1. Domain structure of CIP/KIP proteins. Regions of sequence conservation are indicated by boxes. The thick line after the Cdk inhibitory domains in mouse and human p57 indicated extended regions of sequence conservation. The cyclin-kinase-binding domain contains two subdomains: one that interacts with the cyclin subunit and one that interacts with the Cdk subunit. These regions are indicated by the thick bars. The locations of several anti-p21 antibody-binding sites on p21 are shown by the arrows. The properties of these antibodies are collected in Tables II and III.

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A substrate and association of p107 and p21 with the kinase is mutually exclusive.¹⁷ Residues 38–60 form an α -helical linker that connects the cyclin-binding and Cdk-binding domains in the inhibitor.¹²

A large number of interactions and structural changes are observed in the Cdk2/p27-binding interface. A highly conserved hydrophobic segment of p27 (residues 60–76) forms an amphipathic β hairpin. This subdomain buries a hydrophobic patch in Cdk2 that becomes exposed on p27 binding. The importance of this motif in providing affinity for the Cdk/p27 interaction is indicated by the finding that mutation of D52 in p21 (D63 in p27) to alanine reduces association with Cdk2 by 30-fold.¹⁸ In contrast, mutations of most of the other charged residues has less than a 2-fold effect on binding.¹⁸ Residues 75–81 form an antiparallel β sheet with a segment of the N-terminal domain of Cdk2 (residues 16–22). To accommodate this interaction, an extensive β -sheet network in Cdk2 is disrupted. Residues 85–90 form a β_{10} -helix that binds to the cleft normally occupied by ATP. The most critical residue in this region is Y88, which occupies a position analogous to that of the purine ring in ATP and mimics hydrogen bonds made by the purine ring. Tyrosine-88 as well as residues in the β strand of p27 (W76 and E78) are fully conserved in CIP/KIP proteins (see sequence alignments in Refs. 6 and 7).

CIP/KIP proteins have an additional C-terminal domain that is implicated in association with other proteins (Fig. 1). An 20-amino acid segment at the C terminus of p21 is sufficient to direct association with the trimeric form of proliferating cell nuclear antigen (PCNA)¹⁹ and alanine scanning mutagenesis indicates that the charged residues in this region contribute substantially to binding.¹⁸ The majority of the interaction is provided by the 8-residue segment QTSMTDFY (residues 144–152). M147 and F150 in the segment are critical for PCNA binding.¹⁹ PCNA is required for DNA replication catalyzed by DNA polymerase δ and is found in complexes with p21 in normal diploid fibroblasts.²⁰ *In vitro*, full-length p21 and peptides containing the binding motif can associate with PCNA and block its ability to function in replication by DNA polymerase δ .^{19,21,22} The binding constant for the p21/PCNA interaction (~ 300 nM by surface plasmon resonance)²³ is significantly weaker than the interaction with cyclin-kinase complexes.

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²⁰ Y. Xiong, H. Zhang, and D. Beach, *Cell* **71**, 505 (1992).

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PURIFICATION AND ANALYSIS OF CIP/KIP PROTEINS

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The C-terminal domains of p27 and p57 contain a conserved motif called the QT domain⁷ that is structurally unrelated to the PCNA-binding domain of p21. Previous studies have failed to show association of p27 homologs with PCNA.²¹ Although proteins that can interact with this conserved domain have not been identified, the strong conservation of this domain between p27 and p57 homologs suggests that it functions in protein recognition. Interestingly, *Xenopus* p27^{Kix1} and p28^{Kix1} display features of both p21 and p27. Both inhibitors contain QT-like domains at their extreme C termini and also contain sequences weakly similar to the PCNA binding domain of p21. Consistent with this, *Xenopus* p28^{Kix1} can associate with PCNA *in vitro*, but its affinity is much lower than with p21^{Cip1}.⁸

Unlike p21 and p27, p57 contains an extended spacer region containing repetitive elements between the kinase inhibitory domain and the QT domain (Fig. 1). The function of this domain is unknown. Although the spacer regions of mouse and human p57 have diverged significantly, these two proteins represent the same gene as the mouse gene maps to chromosome 7 at a position that is syntenic with the human gene, which is located at 11p15.5.⁷

Expression and Purification of CIP/KIP Proteins

Several systems have been reported for production of functional CIP/KIP proteins.^{1,3-10} In general, these involve production of fusion proteins in bacteria. The primary advantage of such fusion proteins is the ease of purification. However, some fusions (notably glutathione *S*-transferase, GST) generate a protein with lower specific activity than the unfused protein.¹⁰ This may reflect the use of N-terminal fusions where the GST moiety is adjacent to the kinase inhibitory domain. As such, the use of expression systems that provide either authentic CIP/KIP proteins¹⁰ or fusions containing short His₆ tags^{1,4,6} may be better for most applications.

A major problem with expression and purification of untagged p21 is that it is highly insoluble in bacteria and, under standard induction conditions, is almost exclusively found in inclusion bodies. Although this protein can be purified from denatured inclusion bodies in an active form,¹ it is extensively aggregated and readily precipitates at ca. 1 mg/ml. This is in contrast with p27, which is more soluble (up to 20 mg/ml) and largely monomeric when produced in bacteria (J. W. Harper, unpublished data). Despite the insolubility of p21, it is possible to purify highly active protein, albeit in relatively small quantities, from the soluble fraction of bacteria grown at 20–25°.¹⁰ Purification is greatly simplified by the fact that p21 is heat stable. Thus, even though soluble p21 is a minor component in the bacterial lysate, the majority of bacterial proteins are readily removed by a boiling step. Although not absolutely necessary, this approach also simplifies purification

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TABLE I
EXPRESSION OF PLASMIDS FOR PRODUCTION OF RECOMBINANT CIP/KIP FAMILY MEMBERS^a

Protein	Plasmid	Species	Comments	Reference
Bacteria				
p21 ^{Cip1}	pGEX-2TK	h, m	GST fusion	1, 3
	pRSET	h	His ₆ -gene 10 fusion	1
	pET	h	Authentic p21	10
	pET-HA	h	N-terminal HA tagged	10
	pBS	h, m	IVT/ISH	1, 24
p27 ^{Kip1}	pETHis ₆	h, m	C-terminal His ₆ fusion	4
	pGEX-KG	m	GST fusion	5
	pCRII	m	IVT/ISH	Harper, unpublished, 1994
p57 ^{Kip2}	pGEX-2TK	m	GST fusion	7
	RSET	h	His ₆ -gene 10 fusion	Harper, unpublished, 1995
	pET	h	FLAG-tagged fusion	6
	pBS	h, m	IVT/ISH	7
p28 ^{Kix1}	pET-His ₆	x	His ₆ fusion	8
p27 ^{Xic1}	pGEX-2T	x	GST fusion	9
Insect cells				
p21 ^{Cip1}	pVL1393	h	Authentic p21	3, 10
	pVL1393	h	GST fusion	Harper, unpublished, 1993
p57 ^{Kip2}	pVL1393	h	Authentic p57	Harper, unpublished, 1993
	pBB-His	m	His ₆ -gene 10 fusion	7
Mammalian cells				
p21 ^{Cip1}	Adenovirus	h	Authentic p21	26

^a h, Human; m, mouse; IVT, *in vitro* translation; ISH, *in situ* hybridization. Reagents in References 1, 7, 10, and 24 or listed as unpublished are available either from the American Type Culture Collection (Rockville, MD) or from the laboratory of origin.

of His₆-p27.^{4,10} Plasmids available for expression of CIP/KIPs and for generation of probes for *in situ* hybridization (ISH) are collected in Table I. The use of ISH hybridization provides a facile approach to determining the sites of expression *in vivo*.^{7,24} Methods for expression and purification of CIP/KIP proteins are detailed in the following section.

In addition to expression in bacteria, insect cells can also be employed.^{3,10} This system has been used primarily to analyze association of CKIs with their target cyclin-kinase complexes^{3,7,10,11} and, to date, has not been used for large-scale production and purification of recombinant/proteins. The primary advantage of insect cell expression is the ease with which recombinant proteins can be metabolically labeled with [³⁵S]methionine. This allows analysis of complexes produced by insect cell coinfection.^{3,10,11,17} Another

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advantage of this system is that p21 produced in insect cells, in contrast with bacteria, is largely monomeric as assessed by gel filtration analysis of crude cell lysates (E. C. Swindell and J. W. Harper, unpublished observation).

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Purification of Bacterial CIP/KIP Proteins

Solutions

Lysis buffer: 25 mM Tris-HCl (pH 8.0), 25 mM NaCl, 10% sucrose, 0.1 mg/ml lysozyme, 5 μ g/ml leupeptin, 5 μ g/ml antipain, 1 mM phenylmethylsulfonyl fluoride (PMSF)

Equilibration buffer: 25 mM Tris-HCl (pH 8.0), 25 mM NaCl, 5 μ g/ml leupeptin, 5 μ g/ml antipain, 1 mM PMSF

Wash buffer: 25 mM Tris-HCl (pH 8.0), 200 mM NaCl, 5 μ g/ml leupeptin, 5 μ g/ml antipain, 1 mM PMSF

p21 Elution buffer: 25 mM Tris-HCl (pH 8.0), 400 mM NaCl, 5 μ g/ml leupeptin, 5 μ g/ml antipain, 1 mM PMSF

p27 Elution buffer: 25 mM Tris-HCl (pH 8.0), 200 mM NaCl, 5 μ g/ml leupeptin, 5 μ g/ml antipain, 1 mM PMSF

Column buffer: 25 mM Tris-HCl (pH 8.0), 25 mM NaCl, 5 mM dithiothreitol (DTT), 2 mM EDTA, 5 μ g/ml leupeptin, 5 μ g/ml antipain, 1 mM PMSF

Kinase buffer: 65 mM potassium β -glycerophosphate (pH 7.3), 15 mM MgCl₂, 16 mM EGTA, 10 mM DTT, 1 mg/ml ovalbumin

Denaturing lysis buffer: 6 M guanidine hydrochloride, 20 mM sodium phosphate (pH 7.8), 0.5 M NaCl

Denaturing binding buffer: 8 M urea, 20 mM sodium phosphate (pH 7.8), 0.5 M NaCl

Denaturing wash buffer: 8 M urea, 20 mM sodium phosphate (pH 6), 0.5 M NaCl

Denaturing elution buffer: 8 M urea, 20 mM sodium phosphate (pH 4), 0.5 M NaCl

The following protocols for p21 and p27 have been optimized for both purity and activity of CIP/KIP proteins.¹⁰

p21

1. Transform pET-p21 into BL21(DE3) cells and use a single colony to inoculate 5 ml LB medium containing 0.1 mg/ml of ampicillin. Incubate overnight at 37°, shaking. We have found that storage of transformants for extended periods of time leads to reduced levels of expression.

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2. Inoculate 500 ml of LB containing 0.1 mg/ml of ampicillin with the 5 ml overnight culture of pET-p21/BL21(DE3) and grow at 37° shaking until OD_{600} is 1.0. The culture is then induced with 100 μM isopropylthiogalactoside (IPTG) and grown at 25° for 3-4 hr. An overnight induction can be performed, but can lead to increased proteolytic breakdown products.

3. The culture is then centrifuged at 3500 rpm for 10 min (Beckman J6-MI, 4.2 rotor). The cell pellet is resuspended in 34 ml lysis buffer and incubated on ice for 45 min.

Note: All subsequent steps should be carried out at 4° or on ice.

4. The cell suspension is sonicated 5 \times 12 sec (Heat Systems sonicator Model W200R, continuous mode, large tip) and transferred to a 50-ml Falcon tube.

5. A boiling water bath is set up using 800 ml water in a 1-liter glass beaker. The sonicated cell suspension is then boiled in the water bath for 10 min, transferred to a 35-ml plastic Beckman screw top centrifuge tube, and centrifuged at 13,000 rpm for 20 min (Beckman J-21B, JA-20 rotor).

6. The supernatant is passed over a 1-ml HiTrap Q column (Pharmacia, Piscataway, NJ) (previously equilibrated with equilibration buffer) with the aid of a 50-ml syringe (flow rate at \sim 2 ml/min) and the drop-through is applied to a 1-ml HiTrap SP column (Pharmacia).

7. The column is washed with 10 ml wash buffer at a flow rate of 2 ml/min and p21 is then eluted with 3 ml of p21 elution buffer, collecting 6 \times 0.5 ml fractions in 0.65-ml microfuge tubes. Ten-microliter aliquots of the starting lysate, the HiTrap SP drop-through, the 0.2 M NaCl wash, and each fraction from the elution are subjected to SDS-PAGE and visualized with Coomassie Blue (Fig. 2).



Fig. 2. Elution profile for p21 from a HiTrap SP column. p21 was purified from the soluble fraction of *E. coli* as described in the text and aliquots of fractions of the HiTrap SP elution examined by SDS-PAGE and Coomassie Blue staining. Lane 1, column load; lane 2, HiTrap SP drop-through; lane 3, 0.2 M NaCl wash; lanes 4-9, fractions 1-6 from the HiTrap SP elution.

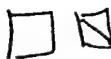
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8. To prevent precipitation of protein and to maximize stability after freeze thawing, 10% (v/v) sterile glycerol is added to eluted column fractions and stored at -70° .



Peak elution fractions (2-4) contain about 400 μ g/ml p21 with an overall yield of 600-800 μ g, as determined by Bradford analysis. This procedure yields a purity of 90-95% with the majority of the contaminating proteins being p21 proteolytic breakdown products, as determined using polyclonal antibodies against p21. Peak fractions contain highly active p21, which can be used for most applications.

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9. When additional purity is required, trace contaminants and most of the p21 breakdown proteins can be removed by high-performance liquid chromatography (HPLC) on a Mono S column. p21 is diluted 1:4 (v/v) with column buffer and applied to a Mono S HR5/5 column (Pharmacia) equilibrated with column buffer at a flow rate of 0.8 ml/min.

10. After washing for 2 min at 100 mM NaCl in column buffer, p21 is eluted with a linear NaCl gradient (100-600 mM) in column buffer over 80 min, collecting 1 min fractions.

11. p21 elutes at 400-450 mM NaCl and is greater than 98% pure by SDS-PAGE.

p27

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The protocol for expression of p27 from pET21a-p27⁴ is identical to that of p21 up to the elution step from the HiTrap SP column. p27 elutes at 200 mM NaCl instead of 400 mM NaCl required for elution of p21.

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1. After loading the HiTrap Q column drop-through onto the HiTrap SP column, the HiTrap SP column is washed with 10 ml equilibration buffer.

2. p27 is then eluted with 3 ml p27 elution buffer, collecting 6 \times 0.5 ml fractions in 0.65-ml microfuge tubes.

Peak fractions, determined by SDS-PAGE, contain about 2 mg/ml of p27 with a greater than 95% purity. Total yield is generally 5-10 mg/liter of starting culture. If additional purity is required, p27 can be further purified by chromatography on a Mono S column using a linear gradient of NaCl (25-300 mM) in 50 min. The protein elutes at 100-150 mM NaCl.

P57

Previous studies have reported the purification of GST-p57⁷ and FLAG-p57⁶ from the soluble fraction of *Escherichia coli* using affinity chromatography on glutathione-Sepharose or using anti-FLAG M2 beads, respectively. In the latter case, p57 is eluted from the column using the FLAG peptide.⁶ Active p57 can also be purified as a His₆ fusion protein

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from the insoluble fraction of *E. coli* after denaturation (E. C. Swindell and J. W. Harper, unpublished data.) The following protocol gives optimal yields and purity of His₆-p57. All steps are performed on ice or at 4°.

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1. Cells from 1 liter of pRSET-hp57/BL21(DE3) are suspended in 70 ml of denaturing lysis buffer and sonicated 5 × 10 sec. The cell suspension is transferred to 35-ml plastic Beckman screw-top centrifuge tubes and centrifuged at 13,000 rpm for 20 min (Beckman J-21B, JA-20 rotor).

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2. Two ml NTA-Ni⁺ beads (Qiagen) are equilibrated in denaturing binding buffer and added to the suspension. The suspension is rotated at 4° for 60 min.

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3. Beads and lysate are transferred to a 10-ml disposable chromatography column (Bio-Rad, Richmond, CA) and the Ni⁺ beads subsequently washed with 50 ml of denaturing binding buffer followed by 50 ml of denaturing wash buffer.

4. p57 is then eluted with 10 ml of denaturing elution buffer, collecting 1 ml fractions. Ten-microliter aliquots are subjected to SDS-PAGE. Peak fractions (3–5) contain ~0.1 mg/ml of p57 with the major contaminant being a 30-kDa protein that reacts with p57 antibodies.

5. Peak fractions are pooled and immediately dialyzed against either 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 10% (v/v) glycerol, or phosphate-buffered saline containing 10% glycerol. p57 is stored in small aliquots at -70°. The yield from 1 liter is 400–600 µg.

Assay of Purified CKIs

The activity of recombinant CIP/KIP proteins should be determined after purification. Previous studies indicate that the K_i values for CIP/KIP proteins and Cdk2 or Cdk4 kinases are in the 0.5–10 nM range while inhibition of Cdc2–cyclin B by p21 is much less efficient ($K_i \sim 400$ nM).¹⁰ Thus, the range of CIP/KIP protein employed will depend on the kinase being assayed. The following procedure applies to Cdk2 and Cdk4 kinases. Although immune complexes from mammalian cell extracts can be used as a source of kinase,¹ activity measurements may be more precisely assayed using purified cyclin–Cdk complexes produced in insect cells. Procedures and reagents for production of such kinase complexes are widely available.^{1,3–10} The use of purified kinase complexes allows an estimate of the concentration of the kinase and cyclin components, which is important when determining the inhibitory activity of the CKI.

1. A 10× dilution series of the CKI to be tested is generated in kinase buffer such that the final inhibitor concentration will range from 0.2 to 100 µM, in twofold increments. Two-microliter aliquots of each dilution is

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added to 17 μ l of a kinase reaction premix containing \sim 0.4 nM cyclin A-Cdk2 and 2.5 μ M histone H1 in kinase buffer. Positive controls lacking CKI and negative controls lacking kinase are prepared in parallel. Reaction mixtures are incubated at room temperature for 10 min.

2. One microliter of 1 mM ATP containing 0.3 μ l of [γ - 32 P]ATP (3000 Ci/mmol) is then added and the mixture is incubated at 37° for 15 min.

The reaction products can be analyzed either by filter binding or by SDS-PAGE.

3. For filter binding, 10 μ l of the reaction mixture is spotted onto phosphocellulose paper (2 \times 2 cm; Whatman, Clifton, NJ P81), allowed to dry, and washed six times with 150 ml of 1% phosphoric acid. 32 P incorporated into histone H1 is determined by liquid scintillation counting. Generally, negative control samples contain less than 500 cpm of 32 P.

4. For analysis by SDS-PAGE, 20 μ l of 2 \times SDS-PAGE loading buffer is added to each reaction mixture and 20 μ l electrophoresed through 12% acrylamide gels. The gel is stained with Coomassie Blue, fixed, dried, and subjected to autoradiography. 32 P incorporation into histone H1 can be determined either by phosphoimager analysis or by cutting out the histone H1 bands and counting by liquid scintillation.

Highly active preparations of CKIs will give half-maximal inhibition of cyclin A-Cdk2 with 1–5 nM CKI

Additional assays that can be performed include binding to PCNA and inhibition of polymerase δ catalyzed DNA replication *in vitro*.^{20,21}

Production of Monoclonal Antibodies against CIP/KIP Family Members

The analysis of CIP/KIP proteins and association with Cdk5 is aided by the availability of specific antibodies that can be used for immunoprecipitation, immunoblotting, and immunohistochemistry. A number of polyclonal and monoclonal antibodies have been generated and some of their characteristics reported.^{1,3–7,10,17} Because of the relatively high degree of sequence identity, particularly in the inhibitory domain, there is potential for antibody cross-reaction. We have noted substantial cross-reaction of some polyclonal anti-p27 antibodies with both p21 and p57 (J. W. Harper, unpublished data). Therefore, when applications involve either immunoprecipitation or immunohistochemistry, it is important to verify that the antibody used is highly specific. Some, but not all, monoclonals may have advantages in this regard. In this section, we describe the preparation and characterization of monoclonal antibodies against p21 and p57.

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p21 Antibodies

In generating p21 antibodies, we chose to use antigen that was purified from the soluble fraction of *E. coli*. In principle, this may generate antibodies that recognize properly folded forms of the protein and therefore may be more applicable to immunoprecipitation procedures. Mice were injected a total of seven times every 3–4 weeks intraperitoneally with approximately 25 µg of p21 per injection (purified through the HiTrap SP step). Intravenous injection (with 30 µg of protein) and fusion were performed using standard procedures.²⁵

Antibodies were screened initially by enzyme-linked immunosorbent assay (ELISA), with 0.3 µg of purified p21 per well. Of the 800 colonies whose culture supernatants were screened, 80 were positive. ELISA-positive antibodies were then tested in three other assays: Western blotting, immunoprecipitation, and coupled immunoprecipitation/kinase assays. Thirty-five of the 80 ELISA positives were also positive by Western blot analysis. In addition, the strongest (as well as some weaker) ELISA positives were screened by immunoprecipitation of [³⁵S]methionine-labeled extracts of insect cells infected with a baculovirus expressing p21. In these extracts, p21 represents a small percentage of the total input proteins, so this is a stringent assay for specificity of immunoprecipitation. Of 39 ELISA positives screened in this way, 27 were positive as judged by immunoprecipitation. Two final criteria were used to determine which antibodies should be clonally selected. First, antibodies that were positive by the latter method were assayed for the ability to immunoprecipitate cyclin-dependent kinase activity from WI-38 whole cell lysates. Although the physiological relevance, if any, is unknown, it has been shown previously that p21 immune complexes from WI-38 fibroblasts or from insect cell coinfections contain significant quantities of histone H1 kinase activity.^{10,11} Nineteen of 24 antibodies were capable of immunoprecipitating varying degrees of kinase activity (Table II).

Based on these analyses, 13 hybridoma wells were subjected to single cell cloning and positive clones used to produce antibody supernatants for further characterization. Monoclonal antibodies were tested for relative reactivity and specificity by immunoblotting WI-38 whole cell lysates as well as bacterial CIP/KIP proteins, immunoprecipitation of p21 with and without associated kinases, and some antibodies were tested for use in

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²⁶ J. A. Eastham, W.-W. Zhang, J. Wang, I. Sehgal, S. J. Hall, T. Timme, G. Yang, L. Connell-Crowley, S. J. Elledge, J. W. Harper, and T. T. Thompson, *Cancer Res.* **55**, 5151 (1995).

²⁷ M. Meyerson and E. Harlow, *Mol. Cell. Biol.* **14**, 2077 (1994).

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TABLE II
MONOCLONAL ANTIBODIES AGAINST p21^{CIP1}

Designation	Isotype	Immunoprecipitation		Immunoblotting ^c	Epitope ^d
		p21 protein ^a	Kinase activity ^b		
CP2	IgG _{2a}	+++	+	++	1-17
CP36	IgG ₁	++	+++	+++	17-30
CP49	IgG _{2b}	+++	+	+	100-164
CP50	IgG _{2b}	+/-	-	+	30-78
CP55	IgG _{2a}	+	NT	+	55-80
CP68	IgG _{2b}	+++	+	+	100-164

^a Immunoprecipitation of p21 was examined using [³⁵S]methionine-labeled p21 generated by expression in insect cells. Immune complexes were subjected to SDS-PAGE and proteins visualized by autoradiography.

^b p21-associated kinase activity in whole cell WI-38 extracts was measured as described in ref. 10 using histone H1 as substrate.

^c Detection of p21 by immunoblotting was accomplished using whole cell lysates from WI-38 cells (30 µg/lane). Detection was accomplished using enhanced chemiluminescence (ECL, Amersham).

^d Epitope mapping was accomplished using immunoblotting with a panel of GST-p21 fusion proteins.

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immunohistochemistry. Finally, antibodies were epitope mapped by screening them against immunoblots with GST fusion proteins containing different portions of p21. These included fusion proteins containing full-length p21, residues 70-164, 1-80, and 70-100. Epitopes recognized by the antibodies were subsequently mapped more finely with smaller portions of p21. A summary of the properties of the resulting 13 monoclonal antibodies is shown in Tables II and III.

Summary of Anti-p21 Antibody Properties

The anti-p21 monoclonal antibodies were grouped into six categories based on epitope mapping experiments and immunoprecipitation of endogenous p21 complexes from metabolically labeled WI-38 fibroblasts (see Tables II and III). To test each antibody, whole cell extracts of these cells were immunoprecipitated using established procedures,²⁰ and Western blots of the immunoprecipitates were probed sequentially with the antibodies listed in Table III. Interestingly, the anti-p21 antibodies immunoprecipitated different subsets of proteins in complexes with p21, and the pattern of polypeptides observed in such complexes correlated to some extent with the epitope recognized by the antibody (Fig. 1). For example, antibodies

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TABLE III
COMPLEXES IMMUNOPRECIPITATED FROM WI-38 CELL EXTRACTS WITH
ANTI-p21 ANTIBODIES^a

Designation	Cdk2	Cyclin A	Cyclin E	Cyclin D1	Cdk4	PCNA
CP2, CP66	+	+	+	+	+	+
CP13, CP68	+	+	+	+	+	+
CP19, CP23, CP36, ^b CP74	+	+	-	-	-	-
CP49, CP59	+	+	+	+	+	+
CP55	-	-	-	-	-	-

^a Whole cell extracts from WI-38 fibroblasts were immunoprecipitated with the indicated anti-p21 antibodies and immune complexes subjected to immunoblotting with antibodies against the indicated cyclins, Cdk2s, and PCNA. Detection was accomplished using ECL. Cyclins A and D were detected with rabbit polyclonal antibodies (W. Harper and L. Zukerberg, respectively). Anti-cyclin E (HE 12), anti-Cdk2 (Santa Cruz), anti-PCNA (Oncogene Science), and anti-Cdk4²⁷ antibodies were also used.

^b This antibody has been shown to perform well in immunohistochemical analysis of p21 expression in both paraffin and frozen tissue sections.

recognizing amino-terminal or carboxy-terminal portions of p21 were able to immunoprecipitate the full complement of proteins previously identified in p21 complexes, including cyclins A, D, and E, as well as PCNA, Cdk2, and Cdk4. Antibodies CP2 and CP68 belong in this class. In contrast, another class of antibodies (exemplified by CP36) was restricted in its ability to precipitate only cyclin A complexes with Cdk2. A third class (CP55) was able to immunoprecipitate p21 to the exclusion of all other previously identified proteins. Consistent with this, CP55 has been shown to significantly reduce the activity of p21 as an inhibitor of cyclin A-Cdk2. The specificity of these antibodies toward different kinase complexes has been confirmed by immunoprecipitation of complexes produced by baculovirus infection of insect cells using different combinations of Cdk and cyclin viruses (J. W. Harper, unpublished data).

CP36 is the strongest antibody identified for immunoblotting and gives approximately 10-fold stronger signals than the other antibodies (Table II). This antibody recognizes an epitope located between amino acids 17 and 30 (Fig. 1). This region is highly conserved between CIP/KIP family members and overlaps the cyclin-binding domain. We have found that CP36 cross-reacts weakly with recombinant p27 and p57 on immunoblots; signals are 50- and 100-fold weaker than with an equal quantity of p21, respectively. In addition, CP36 can immunoprecipitate p27 and p57, but with very low efficiency. None of the other antibodies has been found to cross-react with other CIP/KIP family members. In addition, despite the high degree of conservation between human and rodent CIP/KIP proteins, none of the

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PURIFICATION AND ANALYSIS OF CIP/KIP PROTEINS

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anti-p21 antibodies reacts strongly with the mouse protein, although CP36 recognizes rat p21 in immunoblots and in immunohistochemical assays (G. Darlington, personal communication).)

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Production and Properties of p57 Antibodies

Using analogous procedures, antibodies were generated against His₆-tagged human p57. Of 2000 colonies tested, 128 ELISA-positive antibody supernatants were obtained. The selection of antibodies for single-cell cloning was based on immunoblotting of lysates from insect cells expressing human or mouse p57 and on immunoprecipitation of *in vitro* translated human or mouse p57. A total of five anti-p57 antibodies were selected for single cell cloning and the properties of these antibodies are summarized in Table IV.

All of the antibodies perform well in immunoblot analyses of crude insect cell lysates containing human p57 and immunoprecipitate *in vitro* translated p57. However, only two antibodies were found to react with mouse p57 (KP39 and KP90). Although these two antibodies are useful for immunoblotting mouse p57 in tissue extracts (e.g., placenta), the efficiency of immunoprecipitation of *in vitro* translated mouse p57 is 80- to 90-fold lower than precipitation of human p57 (J. Winston, unpublished data). None of these antibodies has been used successfully for immunohistochemistry. Importantly, none of the p57 monoclonal antibodies cross-reacts with p27 or p21 on immunoblots of recombinant proteins.

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TABLE IV
MONOCLONAL ANTIBODIES AGAINST p57^{KIP2}

Designation	Isotype	Immuno- precipitation ^a		Immunoblotting ^b	
		Human	Mouse	Human	Mouse
KP10	IgG _{2b}	++++	++	+++	+++
KP30	IgG ₁	+++	-	+++	-
KP39	IgG ₁	+++	+	+++	+++
KP42	IgG _{2b}	++++	-	+++	-
KP90	IgG ₁	+++	+	+++	+++

^a Immunoprecipitation was tested using *in vitro* translated and [³⁵S]methionine-labeled p57 from mouse or human. Immune complexes were subjected to SDS-PAGE and visualized by autoradiography.

^b Immunoblotting was performed using mouse of human p57 expressed in insect cells (see Table I for expression vectors used). Detection was accomplished using ECL.

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Further examination of the properties of these antibodies has been hampered by the availability of tissue culture cell lines that express detectable levels of p57 and also contain the full complement of Cdk-cyclin complexes. To date, we have found significant p57 only in HeLa cells, which lack cyclin D-Cdk4 complexes. Anti-p57 antibodies precipitate ~10% of the total Cdk2 in these cells under conditions where p57 is essentially depleted and these p57 immune complexes contain substantial histone H1 kinase activity (5-10% of that contained in a Cdk2 immune complex) (unpublished data).

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Conclusion

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In this chapter, we provide a description of reagents and techniques useful in the analysis of CIP/KIP proteins. Although it is clear that different cyclin-Cdk complexes and a DNA replication factor can associate with p21 during cell cycle progression, the functional outcome of such associations will need further clarification. The availability of specific (non-cross-reactive) antibodies directed against particular epitopes of CIP/KIP proteins may allow distinct Cdk complexes to be isolated and their properties compared *in vitro*. It is conceivable that peptides mimicking the antibody-binding sites on CKIs can be used to facilitate the purification of endogenous p21-containing complexes, allowing the analysis of their properties. Although much less is known about p27 and p57 with respect to their associated proteins in mammalian cells, it is likely that there will be other proteins identified that associate with C-terminal domains of these CKIs. Thus, the expression vectors and antibodies described here should be generally applicable to the analysis of CKI function both *in vitro* and *in vivo*.

Acknowledgments

We thank C. Gorka for initial assistance in the characterization of p21 antibodies. This work was supported by NIH grants to S. J. E., J. W. H., and E. H. B. D. D. is a recipient of a Damon Runyon Scholars Award, a kind gift of E. and K. Langone. E. H. is an American Cancer Society Research Professor. S.J.E. is an investigator of the Howard Hughes Medical Institute.

p53-Dependent Inhibition of Cyclin-Dependent Kinase Activities in Human Fibroblasts during Radiation-Induced G1 Arrest

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Summary

γ -Irradiation of human diploid fibroblasts in the G1 interval caused arrest of the cell cycle prior to S phase. This cell cycle block was correlated with a lack of activation of both cyclin E-Cyclin-dependent kinase 2 (Cdk2) and cyclin A-Cdk2 kinases and depended on wild-type p53. Although the accumulation of cyclin A was strongly inhibited in γ -irradiated cells, cyclin E accumulated and bound Cdk2 at normal levels but remained in an inactive state. We found that both whole-cell lysates and inactive cyclin E-Cdk2 complexes prepared from irradiated cells contained an activity capable of inactivating cyclin E-Cdk2 complexes. The protein responsible for this activity was shown to be p21^{CIP1/WAF1}, recently described as a p53-inducible Cdk inhibitor. Our data suggest a model in which ionizing radiation confers G1 arrest via the p53-mediated induction of a Cdk inhibitor protein.

Introduction

All cells have mechanisms for coping with radiation-induced DNA damage. Most eukaryotic cells respond to such damage with transient delays in both G1 and G2 phases, in the first instance presumably to allow for repair prior to DNA replication or to prevent propagation of damaged cells and in the second instance to allow for repair prior to mitosis (Hartwell and Weinert, 1989). Replication or segregation of damaged DNA is likely to have deleterious consequences for the survival of both the cell and the

organism. This is best demonstrated by mutations that confer defects in cell cycle controls, known as checkpoints. Yeast *rad9* mutations that abrogate the G2 checkpoint control in response to DNA damage render cells sensitive to such damage because chromosome segregation occurs before lesions can be repaired (Weinert and Hartwell, 1988, 1990). Ataxia-telangiectasia (AT) is a human autosomal recessive disorder characterized by hypersensitivity to ionizing radiation (IR; Gatti et al., 1991; McKinnon, 1987). Cells from AT patients have a severely diminished G1 checkpoint control, allowing entry into S phase even under conditions of high DNA damage. Although the molecular basis for checkpoint control in both yeast and human is not well understood, recent reports have identified the nuclear tumor suppressor p53 as a required element of the G1 checkpoint function in mammalian cells (Kastan et al., 1992; Kuerbitz et al., 1992). Fibroblasts from p53 nullizygous mice lack the G1 checkpoint function (Kastan et al., 1992). Furthermore, normal human fibroblasts induce p53 in response to IR whereas fibroblasts from AT patients are defective in this response (Kastan et al., 1992). Finally, the ability of p53 to serve as a transcription factor (Farmer et al., 1992; Funk et al., 1992; Kern et al., 1992) suggests a mechanism for the induction of a number of radiation-responsive transcripts that might influence cell cycle progression (Fornace et al., 1989; Pappathanasou et al., 1991). The recent identification of a p53-inducible cell cycle inhibitor (El-Deiry et al., 1993; Harper et al., 1993; Xiong et al., 1993a; Gu et al., 1993) is consistent with this idea.

The two primary cell cycle transitions, from G1 to S phase and from G2 to M phase, are controlled by a family of related protein kinases known as cyclin-dependent kinases or Cdks (for recent reviews see Meyerson et al., 1992; Pines and Hunter, 1991; Reed, 1992; Xiong and Beach, 1991). Kinase and biological activities require the association of a catalytic subunit and a regulatory subunit known as a cyclin. In mammalian cells, specific catalytic subunits as well as cyclins are required for each phase transition (Lew and Reed, 1992; Sherr, 1993). Regulation of activity appears to be complex, involving both positive (cyclin accumulation [Arion et al., 1988; Desai et al., 1992; Gautier et al., 1988; Labbe et al., 1988]) and negative (phosphorylation of Cdks [Gould and Nurse, 1989; Kumagai and Dunphy, 1991; Morla et al., 1989; Smythe and Newport, 1992]) components. These master cell cycle regulatory elements, then, are likely targets for checkpoint control. It has been demonstrated that DNA damage late in the cell cycle blocks activation of cyclin B-Cdc2, the Cdk that controls the G2/M phase transition (Lock and Ross, 1990; O'Conor et al., 1992). We sought to determine whether IR early in the cell cycle had an impact on the activation of protein kinase activities associated with the G1/S phase transition. Cyclin E-Cdk2 is activated late in G1 and has been implicated in events involving the process of commitment to S phase (Dulić et al., 1992; Hinds

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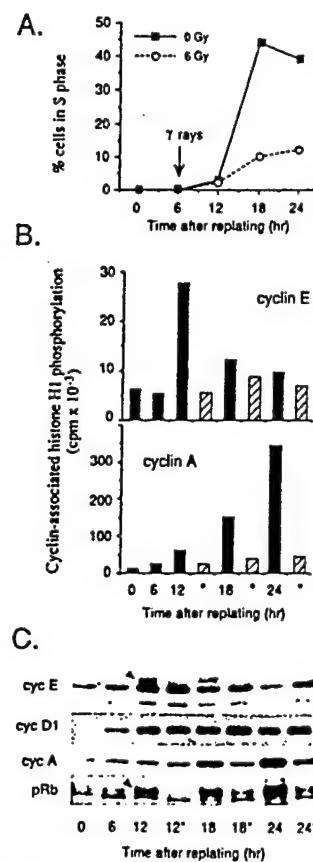


Figure 1. γ -Irradiation in Early G1 Blocks Entry into S Phase and Prevents Accumulation of Cyclin E-Associated and Cyclin A-Associated Kinase Activities in Human Diploid Fibroblasts

(A) Human diploid fibroblast strain NHF1 was synchronized by contact inhibition followed by replating. At 6 hr poststimulation, cells were treated with 6 Gy of γ -irradiation (open circles). The fractions of S phase nuclei were scored based on thymidine incorporation at 6, 12, 18, and 24 hr and compared with unirradiated controls. The solid line shows the kinetic response of unirradiated controls obtained from six independent experiments.

(B) Cyclin-associated histone H1 kinase. Cyclin A and cyclin E complexes were immunoprecipitated from cell extracts prepared from untreated and irradiated (asterisks) NHF1 human fibroblasts at different times after replating and serum stimulation. Kinase activity of the complexes was measured using histone H1 as the substrate as described in Experimental Procedures.

(C) Western blots of the above described cell lysates were immunoblotted sequentially with affinity-purified antibodies to cyclin E and cyclin D1 and antiserum to cyclin A. In addition, the same extracts were used for immunodetection of pRb.

et al., 1992; Koff et al., 1992; Lees et al., 1992; Ohtsubo and Roberts, 1993; Resnitzky et al., 1994). Cyclin A-Cdk2, which appears concomitantly with the onset of S phase, has been shown to be involved in either initiation or maintenance of S phase (Girard et al., 1991; Pagano et al., 1992; Zindy et al., 1992). We found that both activities were strongly inhibited by γ -irradiation. However, the accumulation of cyclin A itself was blocked by IR, while the cyclin E function was blocked apparently by posttranslational mechanisms involving a Cdk inhibitor. Further, we found that the inhibition of Cdks mediated by IR was dependent

upon the ability to accumulate wild-type p53, suggesting that the inhibitor responsible was the recently identified protein p21^{CIP1/WAF1}, shown to be p53 inducible (El-Deiry et al., 1993; Gu et al., 1993; Harper et al., 1993; Xiong et al., 1993a).

Results

IR Impedes Entry into S Phase of Human Diploid Fibroblasts and Prevents Activation of Cyclin E-Associated and Cyclin A-Associated Kinases

Human diploid fibroblast strain NHF1 was synchronized by contact inhibition followed by replating (Kaufmann and Wilson, 1990). At 6 hr poststimulation, cells were treated with 6 Gy (600 rads) of γ -rays. S phase nuclei in irradiated cells were scored based on thymidine incorporation at 6, 12, 18, and 24 hr and compared with unirradiated controls (Figure 1A). Between 6 and 12 hr poststimulation, few cells entered S phase. However, between 12 and 18 hr, 41% of the cells entered S phase in the control compared with 8% of the irradiated cells (81% inhibition). Thus, as has been demonstrated previously, irradiation of fibroblasts during G1 phase blocked entry of most cells into S phase (Little and Nagasawa, 1985).

The inability of γ -irradiated fibroblasts to enter into S phase suggested checkpoint regulation of essential G1 events. In budding yeast, the activation of endogenous Cdk Cdc28 by the accumulation of G1 cyclins has been shown to be essential for the G1 to S phase transition (Richardson et al., 1989). A similar regulatory mode has been proposed for mammalian cells in which the kinases are Cdk2 and Cdk4 and the cyclins are A, D, and E (for review see Sherr, 1993). Therefore, we sought to establish whether these cyclin-associated kinase activities might be targets of negative regulation in response to radiation damage during G1. Quiescent fibroblasts were replated to induce partially synchronous entry into the cell cycle and irradiated at 6 hr poststimulation, as described above. Samples were prepared for analysis of cyclin A- and E-associated kinase activities at the time of replating (0 hr) and at 6, 12, 18, and 24 hr postreplating. Cyclin D-associated kinase activity cannot be measured under these conditions. Based on DNA precursor incorporation data (Figure 1A), the 6 hr timepoint corresponds to G1, the 12 hr timepoint to late G1 near the S phase boundary (G1/S), the 18 hr timepoint to S phase, and the 24 hr timepoint to late S phase and G2. For unirradiated samples (Figure 1B), the cyclin E-associated kinase activity reached maximal levels at the 12 hr timepoint (just prior to S phase), as expected from previous work (Dulić et al., 1992; Koff et al., 1992). Kinase levels then declined as cells progressed through S phase and G2. Cyclin A-associated kinase activity appeared at significant levels first at 18 hr (S phase) and increased as the population became enriched for G2 cells (24 hr). γ -Rays (6 Gy) given at 6 hr after growth stimulation prevented the appearance of both cyclin E-associated and cyclin A-associated kinase activities during the 24 hr time course. Thus, the assembly or activation of the kinase complexes responsible for these

activities are likely targets of G1 checkpoint control in response to damaging irradiation.

IR Does Not Prevent Accumulation of Cyclins D1 and E but Does Inhibit Accumulation of Cyclin A

To determine whether the failure to detect cyclin A- and cyclin E-associated kinase activities in response to γ -irradiation was due to a failure to accumulate the respective cyclins, cyclin proteins were analyzed by immunoblots of cell extracts prepared at the indicated timepoints. In unirradiated cells, cyclin E began to accumulate above the basal G0 level at 6 hr postreplating and reached maximal levels at 12 hr, followed by a decline to baseline levels by 24 hr (Figure 1B) concomitant with the accumulation of cyclin A. Surprisingly, the accumulation of cyclin E was not impaired by γ -irradiation. In irradiated cells, cyclin E accumulated to high levels at 12 hr and persisted at this level through 24 hr. Thus, the failure to activate cyclin E-associated kinase in irradiated cells was not attributable to inhibition at the level of cyclin E accumulation. The accumulation of another G1 cyclin, cyclin D1 (Motokura et al., 1991; Lew et al., 1991; Matsushime et al., 1991; Xiong et al., 1991), which reached maximal levels 12 hr after replating, was also not affected by IR (Figure 1C). In contrast with the effects on cyclins E and D1, accumulation of cyclin A was significantly inhibited by irradiation (Figure 1C), accounting for the low levels of corresponding kinase activity observed.

pRb, the Retinoblastoma Protein, Does Not Become Hyperphosphorylated in γ -Irradiated Cells

It has been shown that the retinoblastoma tumor suppressor gene product (pRb) becomes hyperphosphorylated in the latter part of the G1 interval in human fibroblasts and is maintained in this phosphorylated state for the remainder of the cell cycle. This phosphorylation correlates with the neutralization of the cell cycle inhibitory properties of pRb (Buchkovich et al., 1989; Chen et al., 1989; DeCaprio et al., 1992; Ludlow et al., 1993; Mihara et al., 1989). Recent experimental evidence indicates that cyclin E-Cdk2, cyclin A-Cdk2, and cyclin D-Cdk4 are likely to contribute to the phosphorylation of pRb that occurs in G1 and S phase (Ewen et al., 1993; Hinds et al., 1992; Xiong et al., 1992). Therefore, to determine whether the absence of cyclin E- and cyclin A-associated kinase activities that could be measured was reflected in the phosphorylation state of pRb, we performed immunoblots using pRb-specific antibodies on samples subjected to γ -irradiation and controls, as described above. Hyperphosphorylation of pRb was readily detected by a shift to lower mobility on SDS-polyacrylamide gels. By this criterion, phosphorylation of pRb was dramatically inhibited in irradiated cells (Figure 1C).

IR Does Not Prevent Association between Cyclin E and Cdk2 and between Cyclin D1 and Cdk4

Since accumulation of cyclins D1 and E was unaffected by IR, we sought to determine whether these cyclins in irradiated cells were complexed to their functional partners, Cdk2 and Cdk4, respectively (Dulić et al., 1992; Koff

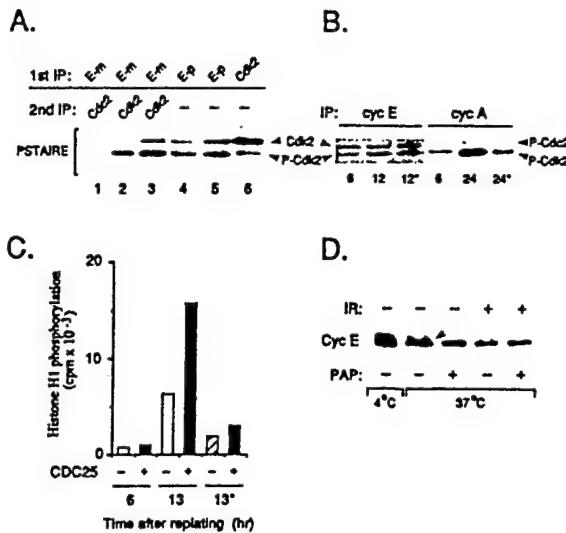


Figure 2. IR Does Not Prevent Cyclin E Association with Cdk2

(A) Cyclin E associates with Cdk2 in human diploid fibroblasts. Cyclin E complexes were immunoprecipitated (IP) from extracts of G1/S phase cells (lanes 1, 2, and 4) and irradiated cells (lanes 3 and 5), as described in Experimental Procedures, using monoclonal antibody (E-m; lanes 1, 2, and 3) or polyclonal rabbit serum raised against bacterially produced cyclin E (E-p; lanes 4 and 5). The complexes were dissociated in 1% SDS and incubated with Cdc2-specific (lane 1) or Cdk2-specific (lanes 2 and 3) antibodies. As a control, Cdk2 was immunoprecipitated directly from the cell extract of asynchronously growing fibroblasts (lane 6). Immune complexes were separated by SDS-polyacrylamide gel electrophoresis (11% gels), transferred onto Immobilon membrane, and detected using anti-PSTAIRE monoclonal antibodies. P-Cdk2, Thr-160-phosphorylated Cdk2.

(B) Cyclin E complexes (cyc E) were immunoprecipitated using monoclonal cyclin E-specific antibody from early G1 (6 hr), unirradiated (12 hr) and irradiated (12' hr) late G1 cells. Cyclin A complexes (cyc A) were immunoprecipitated from early G1 (6 hr), G2 (24 hr) as well as irradiated (24' hr) cells. Immune complexes were separated on 11% SDS-polyacrylamide gels and Western blots were immunoblotted with anti-PSTAIRE antibody as described above. P-Cdc2, Cdc2 phosphorylated on Tyr-15 and Thr-14.

(C) Cdc25 phosphatase cannot activate irradiation-inactivated cyclin E-Cdk2 complex. Cyclin E complexes were immunoprecipitated from the extracts prepared from unirradiated G1 (6 hr) and G1/S-enriched (13 hr) and irradiated (13' hr) cells. The immune complexes were incubated for 30 min at 30°C with bacterially produced glutathione S-transferase (GST)-Cdc25 or with buffer alone and assayed for histone H1 kinase as described in Experimental Procedures.

(D) Posttranslational modifications of cyclin E (Cyc E) are caused by phosphorylation. Cyclin E was immunoprecipitated from the extracts prepared from nonirradiated (minus sign) G1/S-enriched and irradiated (plus sign) cells using monoclonal cyclin E-specific antibodies. Immune complexes were incubated with potato acid phosphatase (PAP) (plus sign) or the buffer alone (minus sign) as described in Experimental Procedures, separated on 11% SDS-polyacrylamide gels, transferred onto the Immobilon membrane, and detected using affinity-purified rabbit cyclin E-specific antibodies.

et al., 1992; Xiong et al., 1992, 1993). To this end, we analyzed cyclin E immune complexes on immunoblots by using anti-PSTAIRE antibody, which detects several members of the Cdk family of protein kinases, including Cdk2 (Figure 2A; Meyerson et al., 1992). We have previously shown that cyclin E immunoprecipitates from HeLa cells analyzed in this fashion contain both Cdk2 and Cdc2, but the associated histone H1 kinase activity depended only

on association with Cdk2 (Dulić et al., 1992). However, the pattern observed from human diploid fibroblasts was consistent with only the two mobility forms of Cdk2 associating with cyclin E (Figure 2A).

When cyclin E immunoprecipitates from irradiated and unirradiated cells (G1/S) were analyzed for Cdk2 association, no significant quantitative or qualitative differences were observed between them: although cyclin E immune complexes from irradiated cells showed less histone H1 kinase activity than those from unirradiated cells, they contained similar amounts of Thr-160-phosphorylated Cdk2 (Figures 2A and 2B). Therefore, reduced cyclin E-associated kinase activity from irradiated cells could not be attributed to failure of cyclin E to associate with the potentially active form of Cdk2. The levels of Cdk2 immunoprecipitated with cyclin A antisera paralleled the levels of cyclin A detected by immunoblotting and the levels of cyclin A-associated kinase shown in Figure 2A.

It has been reported that cyclin-Cdk complexes are subject to inhibitory phosphorylations on Thr-14 and Tyr-15, as has been demonstrated for Cdc2 (Gu et al., 1992; Sebastian et al., 1993). Treatment with the Cdk-specific phosphatase Cdc25, which specifically dephosphorylates these residues, was shown to activate cyclin-Cdk complexes (Gu et al., 1992; Millar et al., 1991; Sebastian et al., 1993). To determine whether this modification might account for the lack of activity of cyclin E-Cdk2 complexes from irradiated cells, immune complexes were treated with bacterially produced Cdc25 and then assayed for kinase activity. Even though Cdc25 treatment had a significant stimulatory effect on cyclin E-Cdk2 activity from nonirradiated G1/S cells (13 hr), cyclin E-Cdk2 complexes from early G1 (6 hr) and irradiated cells (13 hr) remained inactive after treatment (Figure 2C). Thus, cyclin E-Cdk2 complexes from irradiated fibroblasts apparently are not subjected to negative control by phosphorylation at Thr-14 and Tyr-15, which accounts for their inactivity, whereas a significant subset of complexes from nonirradiated G1/S cells are inhibited in this fashion.

Next, we evaluated the association between cyclin D1 and potential partners Cdk4 and Cdk2 under conditions of irradiation. Although implicated in G1 progression (Baldin et al., 1993; Quelle et al., 1993; Resnitzky et al., 1994), cyclin D1 accumulation was not affected by IR (Figure 2B). Cyclin D1 immunoprecipitates contained increasing amounts of Cdk4 and Cdk2 as cells progressed from quiescence to the G1/S phase boundary (data not shown; see Dulić et al., 1993). Thus, irradiation did not interfere with the formation of either cyclin D1-Cdk4 or cyclin D1-Cdk2 complexes.

Activation of Cyclin E-Cdk2 Kinase Correlates with Phosphorylation of Cyclin E

Although IR-induced differences in the level of cyclin E-associated kinase activity did not correlate with levels of cyclin E or with the degree of association of cyclin E with Cdk2, one parameter that did exhibit such a correlation was phosphorylation of cyclin E itself. In extracts from unirradiated cells at times when cyclin E-associated ki-

nase activity was high, 12 and 18 hr poststimulation, a significant fraction of the cyclin E detected by immunoblotting showed reduced electrophoretic mobility (see Figure 1C). These species were not detected in extracts from early G1 cells (6 hr) and were only barely detectable in samples from irradiated cells at any time. Furthermore, the activity detected in immunoprecipitation protein kinase assays across the time course described above correlated well with the levels of the slower migrating cyclin E species rather than the total levels of cyclin E protein. To determine whether changes in electrophoretic mobility were due to phosphorylation of cyclin E, cyclin E immune complexes were treated with potato acid phosphatase or with buffer alone and then analyzed by immunoblotting (Figure 2D). Treatment with acid phosphatase increased the mobility of cyclin E in active samples to that observed in inactive samples, confirming that phosphorylation was responsible for the reduction in mobility observed. However, at this point we have not determined whether this phosphorylation is responsible for cyclin E-Cdk2 activity or is merely a consequence of it.

Irradiation of Fibroblasts Generates (or Maintains) an Activity That Can Inactivate the Cyclin E-Cdk2 Kinase

To investigate the mechanism of IR-mediated inhibition of the cyclin E-Cdk2 kinase, we sought to determine whether lysates prepared from irradiated cells contained an activity that could specifically inhibit this Cdk. Such an activity has recently been observed in epithelial cells treated with the antiproliferative agent transforming growth factor β (TGF β) as well as in extracts of quiescent cells (Slingerland et al., 1994; Polyak et al., 1994) and G1 cycling HeLa cells (Hengst et al., 1994). To this end, active cyclin E immune complexes were incubated with increasing amounts of boiled extracts prepared from cells in early (6 hr) and late (12 hr) G1 phase as well as from the cells that were irradiated 6 hr after replating and then incubated for 6 hr (IR). The boiling step was shown to increase inhibitory activity presumably by releasing inhibitory activity from preexisting intracellular complexes (see below; Hengst et al., 1994). Although all the extracts could inhibit cyclin E-Cdk2 kinase to some degree, the extracts from irradiated cells contained elevated levels of inhibitory activity as compared with lysates prepared from nonirradiated G1/S cells (Figure 3A). Significant amounts of inhibitory activity were also present in extracts prepared from early G1 cells as well as from quiescent cells (Figures 3A and 3C). Further analysis of cyclin E immune complexes treated with inhibitory extracts prepared from irradiated or quiescent cells (see Experimental Procedures) indicated that this *in vitro* inhibition did not involve dissociation of cyclin E-Cdk2 complexes or dephosphorylation of Cdk2 at Thr-160 (Figure 3B). This was in agreement with our observation that IR did not significantly impair association of cyclin E with Cdk2 *in vivo* (see Figure 2). The fact that higher levels of inhibitory activity were detected in lysates from irradiated cells compared with G1 unirradiated cells (6 hr) indicates that the inhibitory activity is induced by IR.

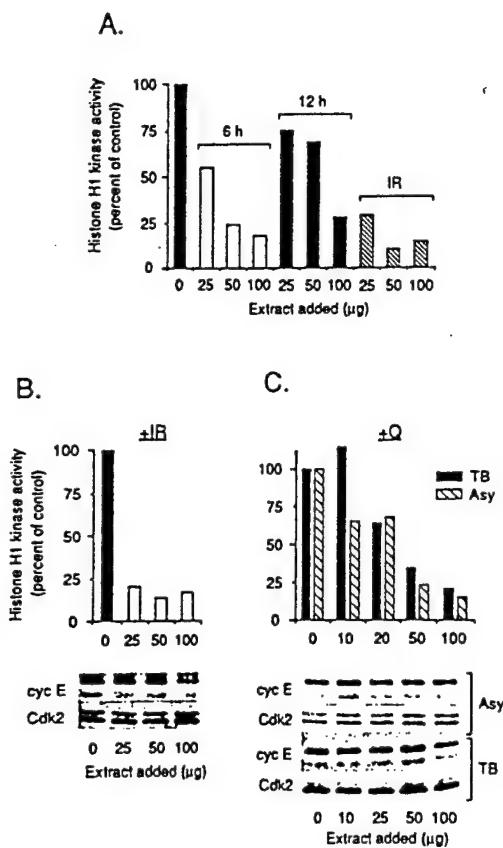


Figure 3. γ -Irradiated and Quiescent Fibroblasts Contain Activity That Inhibits Cyclin E-Associated Kinase

(A) Active cyclin E immune complexes from G1/S phase arrested HeLa cells (equivalent to 150 μ g) were incubated with increasing amounts (25, 50, and 100 μ g equivalents) of boiled cell extracts from G1 (6 hr), G1/S (12 hr), and irradiated (IR) cells for 30 min at 30°C. Complexes treated in this manner were assayed for histone H1 kinase activity. (B) Cyclin E kinase inhibition in the presence of inhibitory extract from irradiated (IR) cells does not involve dissociation of cyclin E-Cdk2 complexes. Cyclin E immune complexes were isolated from asynchronously dividing HeLa cells and treated with boiled extract derived from irradiated fibroblasts. Each sample was then divided, with one half assayed for histone H1 kinase activity and the other separated on 11% SDS-polyacrylamide gels. Western blots were probed with cyclin E-specific monoclonal antibody and anti-PSAIRE monoclonal antibody. (C) Cyclin E kinase inhibition in the presence of inhibitory extract of quiescent (Q) cells does not involve dissociation of cyclin E-Cdk2 complexes. Cyclin E immune complexes from G1/S phase arrested (TB; 200 μ g) and asynchronously dividing (Asy; 400 μ g) HeLa cells were treated with increasing amounts of boiled cell extract from quiescent G0 arrested human diploid fibroblasts (as described above for extracts from irradiated fibroblasts). Note that cyclin E immunocomplexes from G1/S phase arrested cells contained predominantly the Thr-160-phosphorylated form of Cdk2.

The Inhibition of Cyclin E-Cdk2 Kinase Activity in Response to IR Depends on p53

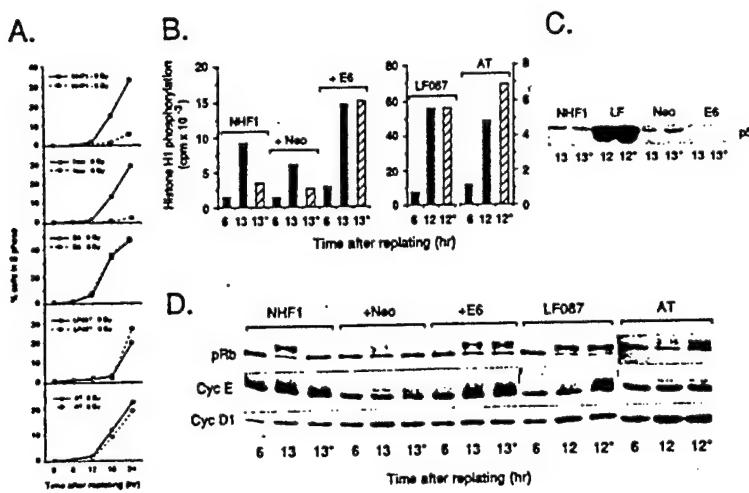
It has been shown that the inhibition of entry into S phase mediated by G1 irradiation of fibroblasts requires the function of the tumor suppressor protein p53 (for review see Prives, 1993). Cells genetically deprived of p53 are incapable of G1 arrest in response to IR (Kuerbitz et al., 1992). Furthermore, p53 protein levels transiently increase upon

exposure to IR (Kastan et al., 1992). Therefore, we determined whether the inhibitory effects on cyclin E-Cdk2 kinase in response to IR were also dependent on p53, which is consistent with the inhibition of this kinase being central to the mechanism of G1 arrest. Fibroblasts rendered deficient in p53 function by three different mechanisms (see Figure 4C for confirmation of p53 status) were subjected to the same radiation protocol employed for normal fibroblasts: fibroblasts homozygous for a naturally occurring germline p53 mutation (Li-Fraumeni syndrome [LF087]), cells derived from patients carrying the recessive genetic disorder AT that fail to increase p53 levels in response to IR (Kastan et al., 1992), and fibroblasts infected with a retrovirus expressing the E6 oncoprotein of human papilloma virus that has been shown to bind and degrade p53 (Kessis et al., 1993; Scheffner et al., 1991). An isogenic control cell line infected with the expression vector alone (plus *neo*) behaved identically to normal diploid fibroblasts in terms of its responses to radiation. Our data demonstrate that cells lacking functional p53 are unresponsive to IR in terms of both the loss of the G1/S irradiation checkpoint (Figure 4A) and the ability to inhibit the cyclin E-Cdk2 kinase (Figure 4B), suggesting that both phenomena are dependent on p53. These results and those reported above are consistent with a model in which the accumulation of p53 in response to irradiation promotes the production or activation of a cyclin E-Cdk2 inhibitor, thus blocking the G1 to S phase transition (see below). Moreover, activation of the cyclin E-Cdk2 kinase in these experiments, as assayed in vitro, always corresponded to in vivo phosphorylation of pRb (Figure 4D), thought to be a critical biological target of the cyclin E-Cdk2 kinase (Hinds et al., 1992). In addition, IR did not prevent cyclin E phosphorylation in p53-deficient cells (Figure 4D).

The IR-Induced Cdk Inhibitor is p21^{CIP1/WAF1}

It has recently been reported that expression of p53 promotes accumulation of a 21 kd Cdk inhibitor known as p21^{CIP1/WAF1} (El-Deiry et al., 1993; Gu et al., 1993; Harper et al., 1993; Xiong et al., 1993a). The p53 dependence on p21 accumulation suggested that it might be identical to the IR-induced Cdk inhibitor detected in our experiments. Therefore, we sought first to determine whether those cells deficient in the accumulation of p53 also did not have elevated levels of Cdk inhibitor in response to IR. Cells expressing the human papilloma virus E6 oncoprotein and therefore having reduced levels of p53 were irradiated and compared with controls for accumulation of Cdk inhibitor activity. Irradiated E6 cells contained considerably lower levels of inhibitor at 12 hr after serum stimulation compared with irradiated controls (Figure 5A). Thus, the accumulation of inhibitor in this system is p53 dependent, as is p21^{CIP1/WAF1}.

To confirm that the Cdk inhibitory activity was authentic p21, we performed immunodepletion experiments with anti-p21 peptide antibodies. First, we showed that the bulk of the inhibitory activity in irradiated cells corresponded to p21. Lysates were incubated with protein A beads to which either anti-p21 or preimmune serum had been cou-



Cyclin E complexes were immunoprecipitated from extracts prepared from unirradiated early G1 (6 hr), G1/S-phase (13 hr) and irradiated cells (13* hr) described above and assayed for histone H1 kinase activity. Note that the scale varies depending on the cell extract used. (C) Western blots (7.5% SDS-polyacrylamide gels) prepared from cell extracts from unirradiated and irradiated NHF1, LF087, Neo, and E6 G1/S phase cells (12 or 13 hr after replating) were probed with p53-specific monoclonal antibody. Note that LF087 cells contain a stabilized mutant form of p53 that accumulates to abnormally high levels and that E6 cells lack p53 protein. (D) Extracts prepared from early G1 (6 hr), unirradiated G1/S phase (12 or 13 hr), and irradiated (12* or 13* hr) fibroblasts were separated on 7.5% SDS-polyacrylamide gels and transferred onto Immobilon membranes. Western blots were probed with monoclonal antibodies to pRb and with affinity-purified polyclonal antibodies to cyclin E and cyclin D1.

pled. Whereas preimmune beads did not remove substantial amounts of inhibitory activity, equivalent amounts of anti-p21 beads removed most of the inhibitory activity (Figure 5B). The remaining inhibitory activity was presumably due to the presence of another recently identified Cdk inhibitor, p28 (Polyak et al., 1994; Hengst et al., 1994), in these lysates. The ability of the anti-p21 beads to remove inhibitory activity, however, was blocked by preincubation with authentic recombinant p21 (Figure 5C). Thus, the radiation induced inhibitor corresponds to p21^{CIP1/WAF1}.

Finally, since we found that the IR-induced Cdk inhibitor was released or activated by boiling, we assumed that it was prebound to cyclin-Cdk complexes in vivo. To test this idea, cyclin D1 and cyclin E immune complexes from both unirradiated and irradiated cells were purified on protein A beads and boiled, and supernatants were assayed for Cdk inhibitory activity. Significant amounts of inhibitory activity were released from the cyclin D1 immune complexes isolated from G1 (6 hr in Figure 5D), G1/S (13 hr in Figure 5D), and irradiated (13* hr in Figure 5D) cells, which is consistent with a significant fraction of the p21 pool being complexed with cyclin D1-Cdk4, as has been reported (Xiong et al., 1992, 1993b). Virtually no inhibitor could be recovered from cyclin D1 complexes from cells expressing E6, although these cells contain normal levels of cyclin D1 (see Figure 4D). Consistent with our direct measurements of kinase activity (see Figure 1), cyclin E-Cdk2 complexes from unirradiated G1/S cells released little inhibitory activity in contrast with those from irradiated fibroblasts, from which inhibitory activity was readily detected (Figure 5D). Most of this inhibitory activity could be immunodepleted using anti-p21 beads, confirming the identity of the inhibitor released from cyclin E complexes

Figure 4. The Inhibition of Cyclin E-Cdk2 Kinase in Response to IR Depends on p53

(A) Normal human diploid fibroblasts (NHF1), fibroblasts infected with the expression vector alone (plus Neo) or retrovirus expressing the E6 oncogene of human papilloma virus, fibroblasts homozygous for a naturally occurring germline p53 mutation (Li-Fraumeni syndrome [LF087]), and fibroblasts derived from patients carrying the recessive genetic disorder AT were synchronized by contact inhibition followed by replating. At 6 hr poststimulation, cells were treated with 6 Gy of γ -irradiation as described in Figure 1A. The fractions of S phase nuclei were scored based on thymidine incorporation at 6, 12, 18, and 24 hr and compared with unirradiated controls. The solid line shows the kinetic response of unirradiated controls, while the broken line shows the kinetic response of irradiated cells.

(B) Cyclin E-associated histone H1 kinase.

as p21^{CIP1/WAF1} (data not shown). Thus, in conjunction with the results presented in Figure 1, these data corroborate our hypothesis that the low activity of cyclin E-Cdk2 complexes present in irradiated fibroblasts results from their association with a Cdk inhibitor, p21^{CIP1/WAF1}.

Discussion

It has been proposed that Cdk activities may control the G1 to S phase transition in mammalian cells. We and others have shown previously that in other cell types, cyclin E and its associated kinase accumulate in late G1 at about the time of commitment to S phase (Dulić et al., 1992, 1993; Koff et al., 1992; for review of the G1 restriction point see Pardue, 1989), suggesting a role in controlling this commitment. Furthermore, it has been shown that deregulation of G1 cyclin levels (both cyclins E and D) by ectopic expression strategies leads to the advance of the G1 to S phase transition, which is consistent with this idea (Hinds et al., 1992; Ohtsubo and Roberts, 1993; Quelle et al., 1993; Resnitzky et al., 1994). Since γ -irradiation of human fibroblasts inhibits entry into S phase, we analyzed whether this treatment affected the accumulation and activation of cyclin E-associated kinase, possibly explaining the failure to initiate S phase. We found that although γ -irradiation blocked the activation of cyclin E-associated kinase, it did not affect either the accumulation of cyclin E or its association with its kinase partner Cdk2. Similarly, neither the accumulation of cyclin D nor the association of cyclin D with its kinase partners was affected. However, the accumulation of cyclin A was strongly inhibited, which accounts for our failure to detect significant levels of cyclin A-associated kinase. The relationship of cyclin A to

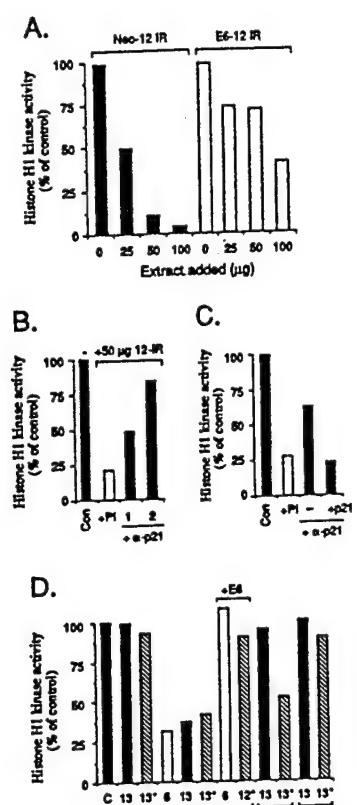


Figure 5. The IR-Induced Inhibitor of Cyclin E-Cdk2 Kinase Activity Is p21^{Cdkn1a}

(A) Fibroblasts expressing the E6 oncoprotein contain little Cdk inhibitory activity. Active cyclin E-Cdk2 complexes were incubated with the indicated amounts of boiled cell extract from irradiated G1/S phase fibroblasts infected with the expression vector alone (Neo-12 IR) or a retrovirus expressing E6 (E6-12 IR) followed by histone H1 kinase activity assay.

(B) Cdk inhibitory activity from irradiated fibroblasts can be depleted with anti-p21^{Cdk/IMAP1} serum. Boiled extracts (equivalent of 50 µg) from irradiated fibroblasts were incubated with 10 µl of protein A beads coupled to preimmune serum (PI) or anti-p21^{Cdk/IMAP1} serum (α -p21; lane 1, 5 µl; lane 2, 10 µl) prior to incubation with immobilized cyclin E-Cdk2 complexes. Histone H1 kinase was assayed as described in Experimental Procedures.

(C) Recombinant p21^{CIP1/WAF1} can block depletion ability of anti-p21^{CIP1/WAF1} beads. Boiled extracts from irradiated cells (equivalent of 20 µg) were incubated with preimmune beads (PI) and with anti-p21 beads (α -p21) that were previously treated with buffer alone (minus) or bacterially produced recombinant p21^{CIP1/WAF1} (see Experimental Procedures).

(D) Cdk inhibitor can be recovered from cyclin D1 and cyclin E immune complexes. Cyclin D1 (α -D1), cyclin E (α -E), and cyclin A (α -A) immune complexes were isolated from the whole-cell lysates prepared from G1 (6 hr), G1/S (13 hr), and irradiated (13 \times hr) NHF1 fibroblasts. In addition, cyclin D1 immunocomplexes were isolated from G1 (6 hr) and irradiated (13 \times hr) fibroblasts infected with a retrovirus expressing the E6 oncoprotein of human papilloma virus (see Experimental Procedures for details). Cyclin complexes were boiled (5 min) and incubated with active cyclin E complexes for 30 min at 30°C prior to histone H1 kinase assay.

cyclins D1 and E is not yet known. However, since cyclins D1 and E accumulate before cyclin A, it is conceivable that either one or both of their associated kinase activities are a prerequisite for accumulation of cyclin A (see below).

p53, Checkpoint Control, and the G1 Restriction Point

The pre-S phase arrest of fibroblasts in response to IR in G1 defines one of the checkpoint controls that may couple genome integrity to cell cycle progression, cell survival, or both (Hartwell and Weinert, 1989). Presumably, damaged nuclear DNA is the signal to which the cell is responding, but the molecular basis for this control is not yet understood. The tumor suppressor gene p53, however, has been implicated in the response to radiation damage (Kastan et al., 1992). p53 is transiently induced in response to irradiation and, as a transcription factor, is thought to induce specific transcripts required for G1/S phase checkpoint control (Kastan et al., 1992). This idea is supported by the observations that fibroblasts lacking a functional p53-encoding allele or depleted of p53 owing to the action of viral oncoproteins, as well as those from patients with AT, which are defective in radiation-induced accumulation of p53, have lost the G1 radiation checkpoint (Kastan et al., 1992). Conversely, ectopic expression of high levels of p53 confers G1 arrest in the absence of irradiation in some cell types (Martinez et al., 1991; Michaelovitz et al., 1990). The point of arrest appears to be late in G1, coincident temporally with the restriction point (Pardue, 1989). Since it has been proposed that Cdks, specifically cyclin E-Cdk2, may be central to restriction point control, we sought to determine whether control of the cell cycle by IR was mediated through regulation of these kinase activities. Our finding that IR blocked activation of the cyclin E-Cdk2 kinase is consistent with this idea. Further, our finding that a lack of p53 or an inability to accumulate p53 abrogated the inhibitory effects of γ -irradiation on cyclin E-Cdk2 activation suggests that p53 is upstream of cyclin E-Cdk2 inhibition in the hierarchy of radiation checkpoint control. These data can now be rationalized in the context of the p53 inducible inhibitor p21^{CIP1/WAF1} and the demonstration that it can be targeted to cyclin E-Cdk2 complexes (El-Deiry et al., 1993; Gu et al., 1993; Harper et al., 1993; Xiong et al., 1993a).

The phenomenology reported here in many respects is similar to what we have observed in human fibroblasts undergoing clonal senescence (Dulić et al., 1993). Interestingly, one of the models recently proposed to account for senescence involves the lack of telomerase activity in cells of limited life span (Greider, 1990; Harley et al., 1990). Eventual loss of telomeric sequences might trigger a DNA damage signal, accounting for the G1 arrest phenotype of senescent cells. Thus, arrest in response to IR and in senescent cells may be functionally related. This notion is further supported by the recent finding that both human fibroblasts and epithelial cells expressing E6 exhibited an extended life span (Shay et al., 1993). Our finding that senescent fibroblasts contain elevated levels of p21 (V. D., G. Stein, and S. I. R., unpublished data) is consistent with this idea.

The Nature of the Cyclin E-Cdk2 Inhibitor

We have shown that cells subjected to IR accumulated an activity capable of inhibiting active cyclin E-Cdk2 complexes. Moreover, we could recover the inhibitory activity

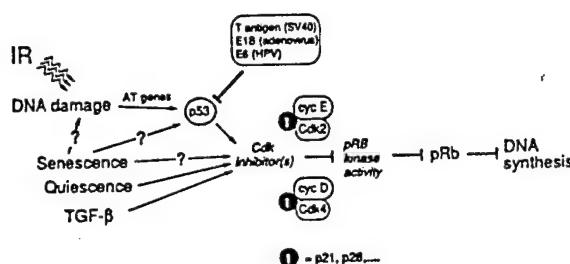


Figure 6. A Model: Inhibitor(s) of Cyclin E-Cdk2 Kinase Activity Can Be Generated by Different Signals Promoting G1 Arrest

IR causes G1 arrest presumably by inducing inhibitor(s) (i) that can antagonize kinase activity of cyclin E (cyc E)-Cdk2 and cyclin D (cyc D)-Cdk4 complexes. As a consequence, unphosphorylated pRb maintains G1 arrest. IR-mediated inactivation of cyclin E kinase is dependent on p53; p53 deficiency impairs this pathway and the G1 block fails to take place. Cells can be arrested in G1/G0 phase by serum deprivation, by contact inhibition, or by both (quiescence); by treatment with TGF β (epithelial cells); and in the case of cellular senescence. Cdk inhibitors are induced under all these circumstances. Note that G1 arrest by cellular senescence differs in many ways from the G1 arrest by quiescence but has similarities to IR-induced arrest. In each of these situations, cells contain moderate to high levels of inactive cyclin E-Cdk2 complexes and fail to phosphorylate pRb. Although similar inhibitory activities have been detected in lysates prepared from cells under these various conditions, it is clear that at least two inhibitory proteins, p21 and p28, are involved. It is not yet clear what the relative contributions of these two inhibitors are under various circumstances.

from cyclin E-Cdk2 complexes isolated from irradiated but not from unirradiated G1/S cells. This inhibitor is identical to a recently characterized inhibitor identified via a two-hybrid screen in yeast for proteins that could interact with human Cdk2 (Harper et al., 1993). p21^{WAF1} was subsequently found to be capable of inhibiting a broad spectrum of cyclin-Cdk activities, including cyclin A-Cdk2, cyclin E-Cdk2, cyclin D-Cdk4, and, to a lesser extent, cyclin B-Cdk1. A cDNA encoding the same inhibitor (WAF1) was simultaneously identified from a subtractive library derived from a p53-transfected tumor cell line (El-Deiry et al., 1993). Based on the observation that the G1 radiation checkpoint is dependent on p53, these authors predicted that p21^{WAF1} would be a critical agent in cell cycle control by radiation. Our results substantiate this mechanistic prediction (Figure 6).

Phenomenologically similar activities have been detected in quiescent and senescent fibroblasts as well as in epithelial cells treated with the negative growth factor TGF β or rendered quiescent by contact inhibition (Slingerland et al., 1994; Polyak et al., 1994). In addition, Cdk inhibitory activities were found to cycle in proliferating cells, accumulating to maximal levels in early to mid-G1 (Hengst et al., 1994). In those cases, it was shown that the inhibitor is a protein and is likely to act via a stoichiometric mechanism, binding directly to Cdk complexes. Several lines of evidence, however, suggest that the predominant Cdk inhibitor identified in many of these situations is not p21 but a different inhibitor (p28) or family of inhibitors. First, inhibitory activity is detected in p53-negative cells, thought not to accumulate high levels of p21 (Xiong et al., 1993b). Second, SDS gel elution experiments suggest that

the inhibitor that accumulates under most circumstances migrates as a 28 kd protein rather than a 21 kd protein (Hengst et al., 1994; Polyak et al., 1994). Thus, it appears that a family of Cdk inhibitory proteins serve as mediators of diverse antiproliferative signals (Figure 6).

In Vivo Distribution of Cdk Inhibitor(s)

The relative recoveries of Cdk inhibitors from cyclin immune complexes suggest that cyclin D1 complexes become saturated at lower levels of inhibitor accumulation than do cyclin E complexes. We did not detect a significant difference in inhibitor released from cyclin D1 complexes prepared from irradiated or unirradiated cells, whereas the amount of inhibitor recovered from cyclin E complexes was significantly increased in irradiated cells. These data suggest that cyclin D1-Cdk complexes may serve as a sink for basal levels of p21, allowing cyclin E-Cdk2 to be activated in unirradiated cells but not in irradiated cells in which inhibitor levels are increased.

Experimental Procedures

Cells, Cell Treatment, and Cell Cycle Analysis

A diploid human fibroblast strain derived from neonatal foreskin (NHF1) was used at passage levels 5–15 (Kaufmann and Wilson, 1990). Cells were grown at 37°C in minimal essential medium (MEM) supplemented with 2 mM L-glutamine, 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μ g/ml streptomycin in a water-saturated atmosphere of 5% CO₂. Proliferation was arrested by growth to confluence. Cell cycling was initiated by replating arrested cultures at 1/4 to 1/5 lower cell density in medium with 10% FBS. LF087 cells were arrested by growth to confluence followed by 1 week incubation in medium with 0.5% FBS. Cell synchrony was assessed by analysis of DNA synthesis activity. Cells were incubated with [³H]thymidine (10 μ Ci/ml; specific activity, 50 Ci/mmol) for 60 min and then fixed with methanol:acetic acid (3:1). After autoradiography, labeled S phase nuclei were enumerated by light microscopy.

Fibroblasts derived from NHF1 and infected with a retrovirus expressing the expression vector alone (plus *neo*) or E6 oncoprotein of the human papilloma virus will be described elsewhere (White et al., 1994). Fibroblasts homozygous for a naturally occurring germline p53 mutation (Li-Fraumeni syndrome [LF087]) were provided by Dr. M. Tainsky, The University of Texas, Houston, Texas (Bischoff et al., 1990; Livingstone et al., 1992; Yin et al., 1992). Fibroblasts derived from patients carrying the recessive genetic disorder AT (strain GM8391) were from the Human Genetic Mutant Cell Repository (Camden, New Jersey) and are described elsewhere (Kaufmann and Wilson, 1994).

Cells were irradiated while attached to dishes and under culture medium using a ¹³⁷Cs γ -ray source with an incident dose rate of 1.4 Gy per minute. Sham-treated (control) cultures were held at room temperature during the interval of irradiation. For further analysis, sham-treated and irradiated cells were harvested by trypsin release at various times after release from confluence arrest. Cells were washed once with Hanks' balanced salt solution and, following sedimentation, frozen at -70°C.

Quiescent human diploid fibroblasts were obtained by growth to confluence with subsequent growth in MEM containing 0.1% FBS for 3 days. Extracts from HeLa cells were prepared as described previously (Dulic et al., 1992).

Reagents

Antibodies to various cyclins and Cdk2 have been described previously (Dulic et al., 1992). In addition to those, we used antiserum specific to the C-terminus of Cdc2 (a gift from C. McGowan, Scripps Institute, La Jolla, California), Cdk2-specific antibody (United Biochemical Incorporated, Lake Placid, New York), and a monoclonal antibody to cyclin E (Dulic et al., 1993). Monoclonal antibody to pRb was purchased from PharMingen (San Diego, California) and to p53 (Ab-6) from Oncogene

Science (Uniondale, New York). Protein A and protein G beads (Gammabind G Sepharose) were purchased from Sigma (St. Louis, Missouri) and Pharmacia LKB (Uppsala, Sweden), respectively.

Potato acid phosphatase was purchased from Boehringer Mannheim (Indianapolis, Indiana). Bacterially produced GST-Cdc25 was a gift from C. McGowan (Scripps Institute, La Jolla, California) and T. Hunter (Salk Institute, San Diego, California; Sebastian et al., 1993).

Rabbit p21^{Cdk1/MPF}-specific antiserum was raised against a peptide corresponding to C-terminal 11 amino acids. For depletion experiments, antibodies from preimmune serum and anti-p21 serum were coupled to the protein A beads (2 ml of antiserum and 100 μ l of protein A beads) using dimethylpimelimidate (Pierce, Rockford, Illinois) as outlined by the manufacturer.

Human p21^{Cdk1/MPF} was obtained by PCR using the following oligonucleotides: 5'-AAGCTTGGATCCTCAGAGGAGGCAGCCATGGCAGAA-3' and 5'-AAGCTTGGATCCTCCTGTGGGGGATTAGGGCTTCCTC-3' and a human HepG2 cDNA library (Schild et al., 1990). To obtain recombinant p21^{Cdk1/MPF} protein for the blocking experiments, we expressed the PCR product cloned into the expression vector pET8C (Studier et al., 1990) in *Escherichia coli* strain BL21. Recombinant p21^{Cdk1/MPF} protein was partially purified from bacterial culture (1 ml) by sonication in lysis buffer (200 μ l) and subsequent boiling (5 min). Thus-treated lysates contained very potent Cdk inhibitory activity (near 100% inhibition of cyclin E-Cdk2 kinase activity with 10 $^{-2}$ μ l). SDS-PAGE analysis showed that p21^{Cdk1/MPF} was one of the major proteins in the boiled bacterial extract. This protein reacted on Western blots with anti-p21 antiserum. Control bacterial lysates did not contain a 21 kd protein and did not exhibit a Cdk inhibitory activity. For blocking experiments, the equivalent of 1 μ l of this lysate was incubated with 30 μ l of p21-beads (or preimmune beads) used for depletion experiments.

All other reagents have been previously described (Dulić et al., 1992, 1993).

Cell Extracts, Immunoblotting, Immunoprecipitations, and Histone H1 Kinase Assays

Extracts were prepared by lysing the cells in ice-cold modified RIPA buffer (50 mM Tris-HCl [pH 7.5]; 150 mM NaCl; 0.5% Triton X-100; 0.5% deoxycholate; 1 mM PMSF; 4 μ g/ml leupeptin, pepstatin, and aprotinin; 1 mM sodium pyrophosphate; 10 mM sodium vanadate; and 25 mM sodium fluoride-mRIPA). When noted, cells were lysed in lysis buffer (LB) containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% NP-40, 1 mM DTT, and protease inhibitors as above. Cell lysate (30–50 μ g) was separated by SDS-PAGE (7.5% gels) and immunoblotting was carried out as described previously (Dulić et al., 1992).

Immunoprecipitation of cyclin complexes from cell extracts (usually 150–300 μ g) and histone H1 kinase assays have been described elsewhere (Dulić et al., 1992). For secondary immunoprecipitations, primary immune complexes were adjusted to 1% SDS and incubated at 37°C for 30 min. Supernatants were then diluted 8-fold with mRIPA buffer and immunoprecipitated with secondary antibody.

Potato Acid Phosphatase and Cdc25 Phosphatase Assays

Cyclin E immunocomplexes were isolated from various cell extracts by using cyclin E-specific monoclonal antibody and were incubated with potato acid phosphatase or with the buffer alone as described previously (Dulić et al., 1992). Proteins were separated by electrophoresis on a 7.5% polyacrylamide gel and immunoblots were probed with affinity-purified rabbit anti-cyclin E antibody.

Treatment of cyclin E and cyclin B1 immune complexes with bacterially produced GST-Cdc25 (or the buffer alone) was according to the protocol described by Millar et al. (1991). Washed immune complexes were then assayed for histone H1 kinase activity.

Kinase Inhibition Experiments

NHF1 cell extracts were prepared by lysing the fibroblasts on the plate with LB. Prior to inhibition assays, appropriate amounts of extract were boiled for 5 min and the precipitate was removed by centrifugation (10 min) in an Eppendorf microfuge. Varying amounts of boiled extract were incubated (28°C for 30 min) with cyclin E immune complexes immobilized on protein G beads isolated from 200 μ g of thymidine block-arrested (G1/S), or from 400 μ g of asynchronous HeLa cell extracts. After incubation, the beads were washed and assayed for histone H1 kinase activity as described (Dulić et al., 1992). Alternatively,

the treated immune complexes were subjected to further analysis by immunoblotting with cyclin E-specific and PSTAIRE-specific monoclonal antibodies.

Inhibitory activity was released from various cyclin-Cdk complexes prepared from 200 μ g of appropriate cell extract, except in the case of cyclin E immune complexes, in which the equivalent of 800 μ g of cell lysate was used because of the low abundance of cyclin E. Cyclin immune complexes were immobilized on protein A or protein G beads and washed several times in lysis buffer prior to boiling. The lysates (~120 μ l) were incubated with 5 or 10 μ l of beads for 30–45 min at room temperature (~20°C) with occasional mixing prior to incubation with immobilized cyclin E immune complexes.

To deplete p21^{Cdk1/MPF}, various boiled cell extracts were incubated (1–2 hr at room temperature) with 10 μ l of protein A beads coupled to either preimmune or anti-p21 antibodies. Extracts treated in this manner were used for Cdk inhibition assays.

For blocking experiments, recombinant p21 was incubated with p21 beads (or preimmune beads) for 2 hr at room temperature. Prior to incubation with cell extracts, the beads were extensively washed to avoid nonspecific binding and subsequent release of p21.

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p57^{KIP2}, a structurally distinct member of the p21^{CIP1} Cdk inhibitor family, is a candidate tumor suppressor gene

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Cyclin-dependent kinases (Cdks) are positive regulators of cell proliferation, whereas Cdk inhibitors (CKIs) inhibit proliferation. We describe a new CKI, p57^{KIP2}, which is related to p21^{CIP1} and p27^{KIP1}. p57^{KIP2} is a potent, tight-binding inhibitor of several G₁ cyclin/Cdk complexes, and its binding is cyclin dependent. Unlike CIP1, KIP2 is not regulated by p53. Overexpression of p57^{KIP2} arrests cells in G₁. p57^{KIP2} proteins have a complex structure. Mouse p57^{KIP2} consists of four structurally distinct domains: an amino-terminal Cdk inhibitory domain, a proline-rich domain, an acidic-repeat region, and a carboxy-terminal domain conserved with p27^{KIP1}. Human p57^{KIP2} appears to have conserved the amino- and carboxy-terminal domains but has replaced the internal regions with sequences containing proline-alanine repeats. *In situ* hybridization during mouse embryogenesis revealed that KIP2 mRNA displays a striking pattern of expression during development, showing high level expression in skeletal muscle, brain, heart, lungs, and eye. Most of the KIP2-expressing cells are terminally differentiated, suggesting that p57^{KIP2} is involved in decisions to exit the cell cycle during development and differentiation. Human KIP2 is located at 11p15.5, a region implicated in both sporadic cancers and Beckwith-Wiedemann syndrome, a familial cancer syndrome, marking it as a candidate tumor suppressor. The discovery of a new member of the p21^{CIP1} inhibitor family with novel structural features and expression patterns suggests a complex role for these proteins in cell cycle control and development.

[Key Words: p57^{KIP2}; p21^{CIP1}; Cdk inhibitor; tumor suppressor; cell cycle]

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The proper development of a multicellular organism is a complex process that requires precise spatial and temporal control of cell proliferation. Cell proliferation in the embryo is controlled via an intricate network of extracellular and intracellular signaling pathways that process growth regulatory signals. This signaling network is superimposed upon the basic cell cycle regulatory machinery that controls particular cell cycle transitions. The ultimate recipient of many of these signals are cyclin-dependent kinases (Cdks), a family of enzymes that catalyze events required for cell cycle transitions.

Cdks require association with cyclins for activation and the timing of Cdk activation is largely dependent on the timing of expression of their cognate cyclin (for review, see Draetta 1993). Several Cdks, including Cdc2, Cdk2, Cdk4, and Cdk6, have been characterized with respect to their temporal activation and their cyclin partners (Matsushime et al. 1992; Meyerson and Harlow 1994; for review, see Pines 1993). Cyclins fall into sev-

eral classes based on their timing of expression and genetic properties (for review, see Sherr 1994). D-type cyclins associate primarily with Cdk4 and Cdk6, whereas cyclin E associates with Cdk2 and both classes appear to function in the G₁/S phase transition. Cyclin A can associate with Cdk2 or Cdc2 and appears to have roles in S phase and G₂. Cyclin F is expressed in late S and G₂, but its kinase partner and role in the cell cycle are not yet known (Bai et al. 1994). Cyclin B binds Cdc2 and controls entry into mitosis. In addition to cyclin availability, Cdks are regulated both positively and negatively by phosphorylation (for review, see Draetta 1993; Solomon 1993).

Once proliferation and morphogenesis have constructed a particular structure, it is of paramount importance that the proliferative state cease and be replaced with a homeostatic state. Although much attention has been focused on how cells enter the cell cycle, little is known concerning the strategies organisms employ to exit the cycle and maintain the nonproliferative state. This state is of great importance to an organism because the vast majority of its cells exist in a nonproliferative

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state throughout adult life. The inability to appropriately halt growth can lead to malformation during development, and to cancer. Thus, equally important in the execution of developmental programs is the arrest of growth once the program is complete. Although the control of terminal differentiation promises to be complex, cell cycle arrest via inactivation of Cdk5 is likely to be a central feature. Recently a new class of Cdk regulatory molecules has emerged that are potential mediators of cell cycle exit and maintenance of the nonproliferative state. These are the tight-binding inhibitors of cyclin-dependent kinases, CKIs. Currently two structurally defined classes of CKIs exist in mammals that are exemplified by p21^{CIP1} (El-Diery et al. 1993; Harper et al. 1993; Xiong et al. 1993; Noda et al. 1994) and p16^{INK4/MTS1} (Serrano et al. 1993; for review, see Elledge and Harper 1994).

p21^{CIP1} is a dual specificity inhibitor that can bind and inhibit G₁ cyclin/Cdk complexes and proliferating cell nuclear antigen (PCNA) (Flores-Rozas et al. 1994; Waga et al. 1994), and can arrest the cell cycle in G₁ when overexpressed (Harper et al. 1995). p21^{CIP1} is transcriptionally controlled by the tumor suppressor protein p53 (El-Diery et al. 1993). It is induced by DNA damage in a p53-dependent manner and is found associated with inactive cyclin E/Cdk2 complexes (Dulic et al. 1994; El-Diery et al. 1994). In some fibroblast cell lines p21^{CIP1} basal expression is dependent on p53; however, in embryonic and adult mouse, p21^{CIP1} expression is independent of p53 (Parker et al. 1995), indicating that the regulation of p21^{CIP1} is more complex than originally thought. p53-Independent expression has also been observed under some circumstances in tissue culture cell lines (Jiang et al. 1994; Michieli et al. 1994; Sheiki et al. 1994). p21^{CIP1} is expressed during embryogenesis primarily in a subset of cells that are mitotic, thus potentially contributing to cell cycle exit during differentiation (Parker et al. 1995). p21^{CIP1} induction has also been observed in cell lines undergoing differentiation *in vitro* (Jiang et al. 1994; Steinman et al. 1994; Halevy et al. 1995; Parker et al. 1995). Thus, organisms utilize p21^{CIP1} in many ways: In proliferating cells in G₁ it can arrest the cell cycle, in S-phase cells it can theoretically block PCNA and slow down DNA synthesis to facilitate repair processes, and in development it may contribute to cell cycle arrest in terminally differentiating cells.

Less is known about the other inhibitors. p27^{KIP1} (Polyak et al. 1994; Toyoshima and Hunter 1994) inhibits both Cdk2 and Cdk4 *in vitro*. It is not regulated by p53 and does not inhibit PCNA (Flores-Rozas et al. 1994), suggesting specialization for a non-checkpoint function. p27 levels show a modest increase in response to conditions that arrest the cell cycle in some cell types (Kato et al. 1994; Nourse et al. 1994). In contrast, p16, p15, and p18, which form a family of structurally related inhibitors consisting primarily of ankyrin repeats, are specific for Cdk4 and Cdk6 (Serrano et al. 1993; Guan et al. 1994; Hannon and Beach 1994; Jen et al. 1994). p16 and p15 have been found deleted in a number of tumor cell lines and in primary tumors and are likely tumor

suppressors (Cairns et al. 1994; Jen et al. 1994; Kamb et al. 1994; Kato et al. 1994; Nobori et al. 1994; Spruck et al. 1994). p15 is induced by TGF- β and is a likely mediator of its growth-arresting properties (Hannon and Beach 1994).

Although considerable information has accumulated concerning the existence of cyclin kinase inhibitors, little is known about the extent of these families, their structural diversity, and potential roles in development. In this paper, we describe the discovery of a new Cdk inhibitor of the p21^{CIP1} family, p57^{KIP2}, that has several unique structural features, patterns of expression during development, and is located in a chromosomal region implicated in a number of human cancers.

Results

Isolation of the cDNA encoding KIP2, cyclin kinase inhibitor protein 2

To identify potential regulators of cyclins and cyclin-dependent kinases, we utilized an improved version of the two-hybrid system (Durfee et al. 1993) that we and others have used previously to identify proteins that bind these molecules (Harper et al. 1993; Toyoshima and Hunter 1994). To screen for mouse cDNAs encoding proteins able to interact with cyclin D, Y190/pAS2-cyclin D1 was transformed with a pACT-mouse embryonic cDNA library, and transformants were subjected to selection for histidine prototrophy on SC-his⁻-trp⁻-leu plates containing 50 mM 3-amino-triazole as described previously. Transformants (5×10^5) were placed under selection, and rare surviving colonies screened for their ability to produce β -galactosidase. Plasmids recovered from 25 His⁺ blue colonies were sequenced from their 5' ends and their deduced amino acid sequences were compared to GenBank. Three of these clones were related to one another and shared structural homology with p21^{CIP1} and p27^{KIP1}. The DNA and deduced amino acid sequence of the protein encoded by the longest cDNA (1.5 kb) is shown in Figure 1A. It encodes a protein of 335 amino acids with a number of unusual structural features. The new inhibitor gene isolated in this study will be referred to as KIP2 because it is most closely related to KIP1, and the protein as p57^{KIP2} based on its size (see below).

The KIP2 protein has a number of unusual structural features

KIP2 has four distinct amino acid sequence domains that will be referred to as domains I-IV for convenience of discussion (Fig. 1C). These are not to be confused with protein structural domains, although they may eventually coincide once structural data are available. Domain I bears significant similarity to p21^{CIP1} and p27^{KIP1} in a region that has been shown to be necessary and sufficient for Cdk inhibition (Polyak et al. 1994; Harper et al. 1995) (Fig. 1D). Domain II is proline-rich region (25% proline) that has a series of alternating prolines interspersed with nonrepetitive sequence. Domain III has an

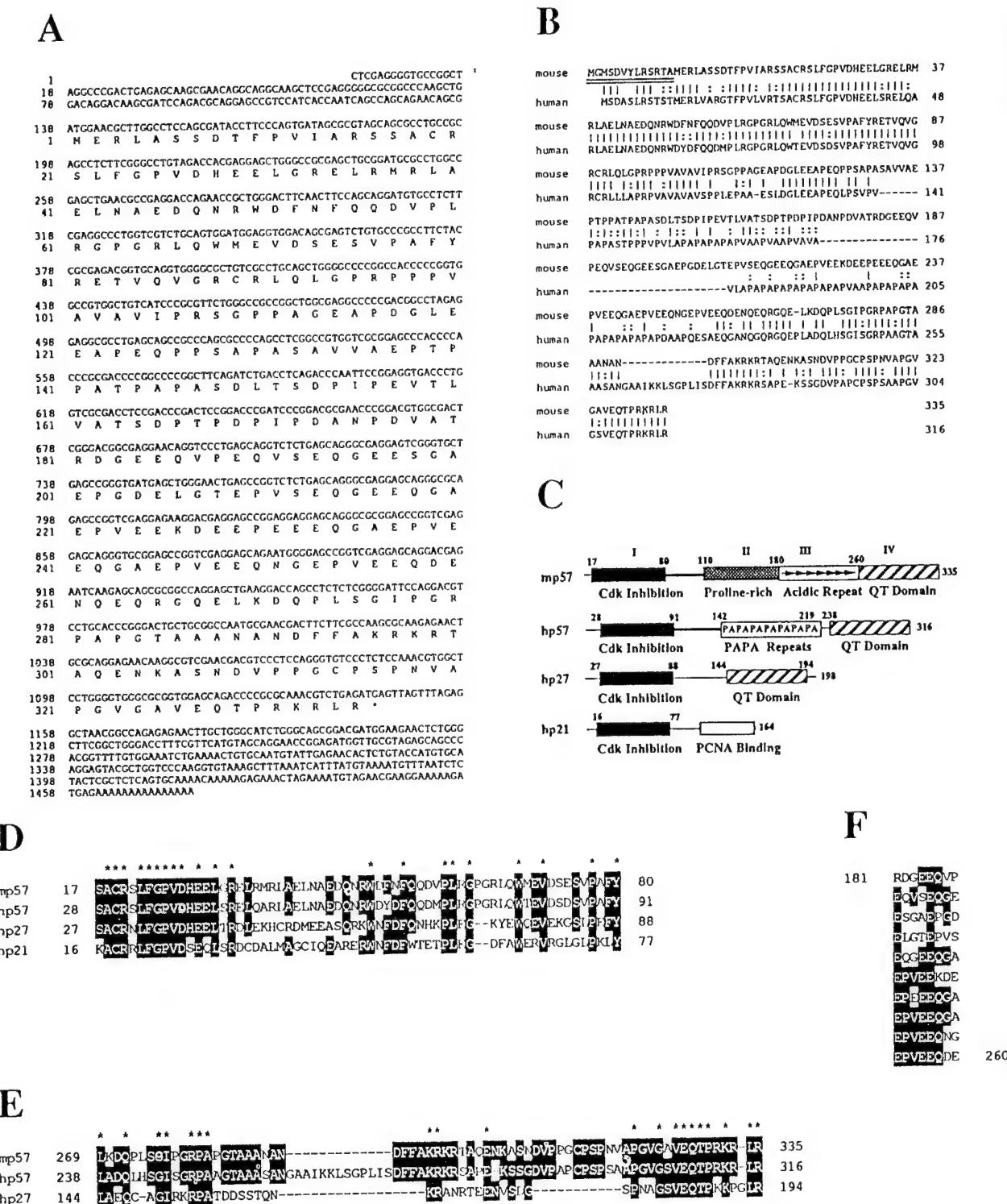


Figure 1. (A) Nucleotide and deduced amino acid sequence of mouse p57^{KIP2}. (B) Sequence alignment of mouse and human KIP2. The amino-terminal 13 amino acids of mouse p57^{KIP2} (underlined) is derived from genomic sequences. (C) Domain structure of the p21^{CIP1} family of CKIs. Domains are indicated by roman numerals. Regions of sequence conservation are indicated as boxes. The thick line after the Cdk inhibitory domain of mp57 and hp57 indicates additional conservation that extends beyond the Cdk inhibitory domain. The extent of the PCNA-binding domain has not been determined precisely. (D) Sequence alignments of the amino-terminal Cdk inhibitory domains of mouse p57^{KIP2}, human p57^{KIP2}, human p27^{KIP1}, and human p21^{CIP1}. Black boxes indicate identical residues shared by at least three sequences; asterisks indicate complete conservation. (E) Sequence alignments of the carboxy-terminal QT box. (F) Alignment of the glutamate repeat sequences of mouse p57^{KIP2}. The GenBank accession number for mouse KIP2 is U22399, and for human KIP2 is U22398.

unusual acidic repeat in that every fourth amino acid is a glutamic or aspartic acid. There is also a slightly degenerate higher order repeat motif of 8 amino acids, EPVEEQXX, shown in Figure 1F. Domain IV has sequence conservation with the carboxyl terminus of p27^{KIP1} (Fig. 1E). The center of the most conserved stretch of sequence identity has the amino acids QT, and we will refer to this conserved motif as the QT box. The carboxy-terminal region of p27^{KIP1} is distinct from the carboxy-terminal region of p21^{CIP1}, which is involved in PCNA binding. Sequence conservation with p27^{KIP1} indicates that the QT box is a structural motif that is likely to function in protein-protein interaction.

The human KIP2 homolog, hKIP2, was isolated by low stringency hybridization screening of a human embryonic cDNA library in λ gt10. Two cDNAs were recovered and sequenced. The deduced amino acid sequence of one of these revealed a protein of 316 amino acids and is shown in Figure 1B aligned with mKIP2. hKIP2 shares two of the four sequence domains of the mouse gene. It has highly conserved the amino- and carboxy-terminal domains; however, it has replaced the internal domains II and III with a distinct region consisting primarily of an alternating proline-alanine repeat termed the PAPA repeat (Fig. 1B,C). The second hKIP2 cDNA contains amino acids 1-96, deletes the entire PAPA region, and is spliced back in at nucleotide 1076 in the +1 reading frame. Thus, the protein potentially encoded by this smaller cDNA consists primarily of the Cdk inhibitory domain. Whether this cDNA represents a normal splicing variant remains to be determined.

The amino acid sequence alignment derived from the longest mouse and human cDNAs revealed that the human cDNA encodes an extra 13 amino acids at its amino terminus. Limited sequence analysis of the mouse genomic region directly 5' of the initiating methionine (P. Zhang and S.J. Elledge, unpubl.) revealed further amino acid sequence identity with the amino terminus of the human cDNA. These additional 13 amino acids are underlined in the sequence alignment (Fig. 1). The difference between the human and mouse cDNAs and the correspondence of the human cDNA and mouse genomic sequence suggest that there may be a mouse cDNA with the 5' end corresponding to the human cDNA. Thus, the difference between the 5' ends may result from alternative splicing. However, analysis of the genomic DNAs indicates that the difference between the internal domains does not result from alternative splicing (P. Zhang and S.J. Elledge, unpubl.).

p57^{KIP2} can bind to cyclin/Cdk complexes in a cyclin-dependent manner

To examine the association of p57^{KIP2} with cyclin/Cdk complexes, in vitro-translated [³⁵S]methionine-labeled mouse p57^{KIP2} was incubated with [³⁵S]methionine-labeled Cdks or cyclin/Cdk complexes produced in insect sf9 cells, and association was determined by immunoprecipitation with anti-Cdk antibodies. p57^{KIP2} was found to efficiently bind Cdk2, Cdk3, and Cdk4 in a

cyclin-dependent manner (Fig. 2A). p57^{KIP2} showed a weaker association with Cdk6/cyclin D2 complexes and no detectable association with Cdk7/cyclin H complexes.

Because p57^{KIP2} was identified in a two-hybrid screen using cyclin D, it is possible that it associates with cyclin/Cdk complexes via direct interaction with cyclins. This is consistent with the fact that association with Cdks is cyclin dependent (Fig. 2A). Furthermore, it has been reported previously that p27^{KIP1} can associate directly with in vitro-translated cyclin D (Toyoshima and Hunter 1994). p57^{KIP2} was tested for its ability to bind cyclins D1, D2, and A in the absence of Cdk subunit using cyclin and p57^{KIP2} proteins produced and [³⁵S]methionine labeled in sf9 cells. Because insect cells contain endogenous Cdks, association of cyclins with p57^{KIP2} could potentially be mediated by endogenous Cdks such as Cdk2 and Cdc2 present in crude sf9 extracts. To reduce the levels of these Cdks and associated cyclins, extracts were precleared with immobilized p13^{sucl+}, which is known to bind both monomeric and cyclin-associated Cdks with high affinity. Using such lysates, association between cyclins and p57^{KIP2} was not observed under conditions in which ~5% binding could be detected (Fig. 2B). Similar results have been obtained using p21^{CIP1} and p27^{KIP1} (Harper et al. 1995). Thus, if contacts are made with cyclins, they are very weak. In this situation, the cyclin dependency could result from the integration of weak interactions with surfaces on both cyclins and Cdks. Alternatively, the cyclin dependence could result from conformational changes on the Cdk or cyclin induced upon formation of the cyclin/Cdk complex that favor p57^{KIP2} binding. It is also likely that the endogenous Cdk in yeast, Cdc28, contributed to the interaction between cyclin D and p57^{KIP2} detected in the two-hybrid system.

p57^{KIP2} can inhibit cyclin/Cdk activity

Having established in vitro association of p57^{KIP2} with cyclin/Cdk complexes, we wished to examine the possible biochemical consequences of this association. Proteins that associate with cyclin-dependent kinases could influence the activity or substrate specificity, modify interactions with other proteins, or alter subcellular localization. We have shown previously that the structurally related protein p21^{CIP1} is an inhibitor of Cdk activity (Harper et al. 1993). To examine whether the p57^{KIP2} alters Cdk function, kinase activities of several cyclin/Cdk complexes were measured in the presence of GST-KIP2 using histone H1 or Rb as substrate (Fig. 2C). p57^{KIP2} is an efficient inhibitor of cyclin E/Cdk2, cyclin A/Cdk2, cyclin E/Cdk3, and cyclin D2/Cdk4 kinase complexes. It shows considerably less activity toward cyclin B/Cdc2 and cyclin D2/Cdk6 complexes. The reduced ability of p57^{KIP2} to inhibit Cdk6 complexes is consistent with the weak association observed in binding assays (Fig. 2B). A potential caveat is that the assays for Cdk6 were performed on cyclin D2/Cdk6 immune complexes, unlike the assays for other kinases, and it is

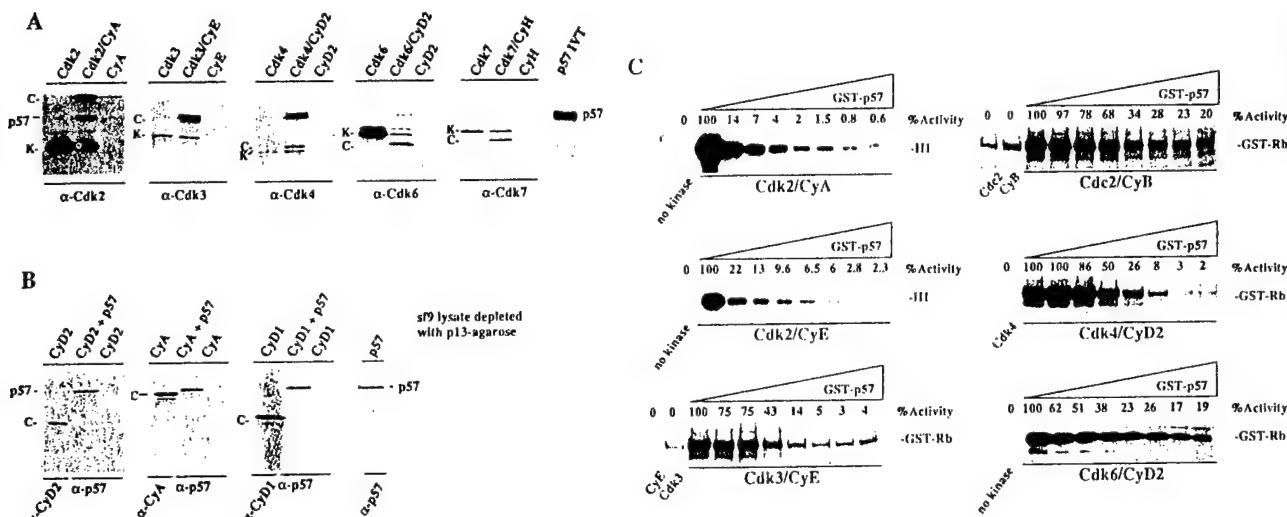


Figure 2. p57^{KIP2} binds and inhibits Cdk family members in vitro. (A) Cyclin-dependent association of p57^{KIP2} with Cdks. [³⁵S]Methionine-labeled sf9 extracts prepared from cells infected with baculovirus expressing a cyclin or Cdk alone, or coinfecting with the indicated cyclin/Cdk pairs were mixed with [³⁵S]methionine-labeled mouse p57^{KIP2} produced by in vitro translation and Cdk-associated proteins isolated by immunoprecipitation of the Cdk subunit. Immune complexes were separated by SDS-PAGE and analyzed by autoradiography. (B) The position of the cyclin component; (K-) the kinase component. CyA is a GST fusion protein. Cdk3 is a T7 gene10 fusion protein and is immunoprecipitated with anti-T7 Tag antibodies (Novagen). Cdk7 contains a carboxy-terminal hemagglutinin A (HA) tag and was immunoprecipitated with anti-HA antibodies. (C) p57^{KIP2} does not directly associate with CyA, CyD1, or CyD2. [³⁵S]Methionine-labeled sf9 lysates for the indicated cyclins were precleared with p13 beads and then incubated with or without T7 Tag-p57 and immune complexes isolated using either antibodies directed against the cyclin or anti-T7 Tag to immunoprecipitate p57^{KIP2} and any associated proteins. As a control for nonspecific binding of the antibodies, cyclin extracts alone were also immunoprecipitated with anti-T7 Tag antibodies. Immune complexes were separated by SDS-PAGE and analyzed using a Molecular Dynamics PhosphorImager. (C) Inhibition of Cdk activity by mouse GST-p57. Inhibition of cyclin A/Cdk2 and cyclin E/Cdk2 [at ~0.4 nM] was examined using kinase complexes purified from insect cells as GST fusion proteins employing histone H1 as substrate. Inhibition of Cdc2/cyclin B, Cdk3/cyclin E, and Cdk4/cyclin D2 was examined using crude insect cell lysates (~1–5 nM in kinase) and 1 μM GST-Rb as substrate [Matsushima et al. 1992; Harper et al. 1993]. Cdk6/cyclin D was assayed using anti-Cdk6 immune complexes from insect cells coexpressing Cdk6 and cyclin D2. The concentrations of GST-p57 used were 0, 1.3, 2.7, 5.3, 10.6, 21.3, 42.5, and 85 nM except for Cdc2/cyclin B where the concentrations were 0, 2.7, 5.3, 10.6, 21.3, 42.5, 85, and 170 nM. Counts incorporated into GST-Rb or histone H1 were quantitated using a Molecular Dynamics PhosphorImager. Counts present in the Cdk or cyclin alone lanes were subtracted from total counts in reactions using Cdk/cyclin complexes to calculate percent activities.

possible that the presence of antibodies could interfere with p57^{KIP2} function. Regardless, it is clear that it is a potent inhibitor of cyclin-dependent kinase activity and shows preference for cyclins and Cdks involved in the G₁ to S-phase transitions.

p57^{KIP2} can associate with Cdk2 in vivo

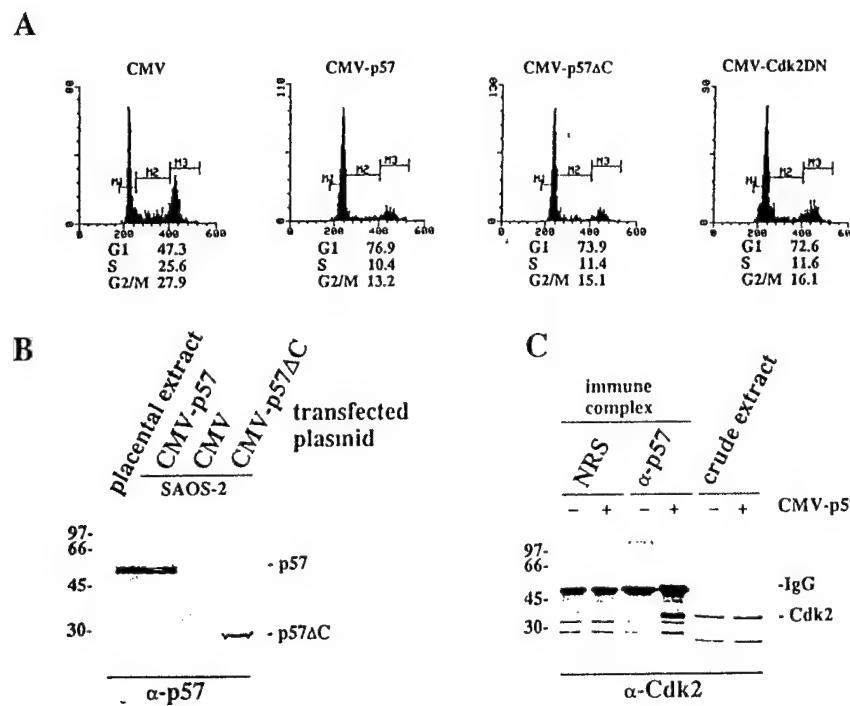
To establish that p57^{KIP2} can associate with cyclin-dependent kinases in vivo, extracts prepared from SAOS-2 cells transfected with either pCMV-p57 or pCMV (negative control) were immunoprecipitated with either anti-p57 or normal rabbit sera and immune complexes subjected to immunoblotting using anti-Cdk2 antibodies. Cdk2 protein was present only in cells transfected with pCMV-p57 and immunoprecipitated with anti-p57^{KIP2} antibodies (Fig. 3C). The presence of Cdk2 in the p57^{KIP2} immunoprecipitates confirms the in vitro binding analysis and demonstrates that p57^{KIP2} can bind to Cdks in vivo.

SAOS-2 cells transfected with mouse pCMV-p57 specifically express KIP2 protein that migrates as a doublet

of 57 kD as detected with anti-p57^{KIP2} affinity-purified antibodies (Fig. 3B). As a control, endogenous mouse KIP2 was detected in placental extracts by Western blot. Placental extracts were chosen because placenta express high levels of p57^{KIP2} mRNA (see below). The placental protein also migrates as a doublet but slightly more slowly than p57^{KIP2} from transfected cells. This may be due to the fact that the transfected cells are expressing the mouse KIP2 cDNA whose protein begins at amino acid 14 of the human cDNA (amino acids MERL). The slight difference in mobility could be explained if the placental form of the KIP2 mRNA has an additional 13 amino acids at the amino terminus like the human cDNA (see Fig. 1). Furthermore, human p57^{KIP2} made by in vitro translation of the full-length cDNA produced a protein that comigrated with the faster migrating form of the mouse p57^{KIP2} (data not shown).

Overproduction of p57^{KIP2} leads to G₁ arrest

To investigate whether p57^{KIP2} can arrest the cell cycle, we utilized a transient transfection assay (Zhu et al.



tein. Proteins were transferred to nitrocellulose and immunoblotted using affinity-purified p57^{KIP2} antibodies, and visualized by ECL (Amersham) detection with an exposure time of 10 sec. The positions of molecular mass markers are shown at left. The size of the p57^{ΔC} protein (29 kD) is consistent with the size of the truncated protein observed after in vitro translation (data not shown). (C) p57^{KIP2} expressed in SAOS-2 cells associates with Cdk2. Whole-cell extracts from SAOS-2 cells transfected with either pCMV-p57 or pCMV (negative control) were immunoprecipitated with either anti-p57 or normal rabbit sera and immune complexes subjected to immunoblotting using anti-Cdk2 antibodies. As controls, 10 μ g of extracts from pCMV-p57 and pCMV-transfected cells were also subjected to immunoblotting with anti-Cdk2 antibodies. The exposure time for ECL detection was 10 sec.

1993). By this approach, it was shown previously that dominant-negative forms of Cdk2 and Cdk3 can block cells in G₁ (van der Heuvel and Harlow 1993) and that overexpression of p21^{CIP1} and p27^{KIP1} cause G₁ arrest (Toyoshima and Hunter 1994; Harper et al. 1995). Expression plasmids for mouse KIP2 expressing full-length p57^{KIP2}, pCMV-p57, or a version expressing the first 167 amino acids, pCMV-p57^{ΔC}, were transfected into SAOS-2 cells along with a plasmid expressing the cell surface marker CD20, and after 48 hr the DNA content of cells expressing high levels of CD20 was measured by flow cytometry (Fig. 3A). SAOS-2 cells are defective for p53 and Rb and have been characterized extensively with respect to cell cycle arrest by both Cdk2-DN (dominant negative) and Rb, both of which lead to predominantly a G₁ arrest (Hinds et al. 1992; van der Heuvel and Harlow 1993). SAOS-2 cells also accumulate in G₁ when transfected with pCMV-p57 (47% vs. 77% in G₁) or pCMV-p57^{ΔC}, indicating that the carboxy-terminal domains are not required for arrest. The overall distribution of cell cycle phases in p57^{KIP2}-arrested cells is similar to cells arrested with Cdk2-DN (Fig. 3A), consistent with the fact that p57^{KIP2} preferentially inhibits Cdk2s involved in the G₁/S transition. These data indicate that p57^{KIP2} alone can function to arrest the cell cycle in G₁ and does not require p53-regulated proteins or Rb.

Figure 3. p57^{KIP2} can associate with Cdk2 in vivo and cause G₁ arrest. (A) SAOS-2 cells were transiently transfected with 2 μ g of pCMVCD20 and 20 μ g of either pCMV, pCMV-p57, pCMV-p57^{ΔC}, or pCMV-Cdk2DN using the calcium phosphate method. Forty-eight hours after removal of the precipitate, cells were stained for CD20 and DNA content, and analyzed by flow cytometry. In the histograms shown, DNA content is shown on the abscissa and cell number on the ordinate. The percentages of cells in G₁, S, and G₂/M are given below each histogram. pCMV-p57^{ΔC} expresses an amino-terminal fragment of mouse p57 (residues 1-167). pCMV-Cdk2DN is a dominant-negative Cdk2 mutant (described previously by van der Heuvel and Harlow 1993), which is used here as a positive control for G₁ arrest. (B) Expression levels of p57 and p57^{ΔC} in transfected cells as determined by immunoblotting. Whole-cell lysates (10 μ g) from SAOS-2 cells transfected with pCMV, pCMV-p57, and pCMV-p57^{ΔC} were separated by SDS-PAGE along with 100 μ g of placental extract as a positive control for p57^{KIP2} protein. Proteins were transferred to nitrocellulose and immunoblotted using affinity-purified p57^{KIP2} antibodies, and visualized by ECL (Amersham) detection with an exposure time of 10 sec. The positions of molecular mass markers are shown at left. The size of the p57^{ΔC} protein (29 kD) is consistent with the size of the truncated protein observed after in vitro translation (data not shown). (C) p57^{KIP2} expressed in SAOS-2 cells associates with Cdk2. Whole-cell extracts from SAOS-2 cells transfected with either pCMV-p57 or pCMV (negative control) were immunoprecipitated with either anti-p57 or normal rabbit sera and immune complexes subjected to immunoblotting using anti-Cdk2 antibodies. As controls, 10 μ g of extracts from pCMV-p57 and pCMV-transfected cells were also subjected to immunoblotting with anti-Cdk2 antibodies. The exposure time for ECL detection was 10 sec.

KIP2 transcription is not regulated by p53

p21^{CIP1} is transcriptionally regulated by p53. To examine whether KIP2 shares this regulation, we employed Rat A1-5 cells, which contain a temperature-sensitive p53 gene (Martinez et al. 1991). A1-5 cells can grow at 39.5°C when p53 is inactive, but cells arrest in G₁ at 32.5°C when p53 becomes active. We examined whether KIP2 mRNA expression increased when p53 became active. Although CIP1 expression increased upon incubation at 32.5°C, KIP2 mRNA remained constant (Fig. 4B). Thus, p57^{KIP2} is not regulated by p53 under conditions in which p21^{CIP1} transcription is induced in these cells.

p57^{KIP2} shows tissue-specific patterns of expression

In contrast to p21^{CIP1} and p27^{KIP1}, p57^{KIP2} mRNA was detectable in only a subset of adult mouse and human tissues. In general, the mouse expression pattern correlates well with the human distribution. p57^{KIP2} is expressed in heart, brain, lung, skeletal muscle, kidney, pancreas, and testis by Northern analysis (Fig. 4) and by *in situ* hybridization (see below). It is expressed most highly in placenta and has low levels in liver and is undetectable in spleen (human spleen has not been tested). Furthermore, although the major transcript is ~1.7 kb,

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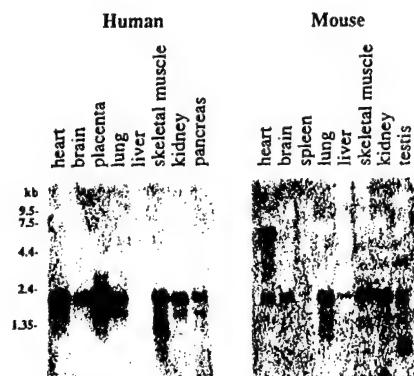


Figure 4. Northern analysis of human and mouse *p57^{KIP2}*. (A) Tissue specificity of KIP2 expression. Blots containing the indicated polyA⁺ RNA (2 μ g per lane) from the indicated tissues were probed with human or mouse *p57^{KIP2}* cDNAs as described in Materials and methods. The human and mouse probes consisted of the entire cDNA. Exposure times were 15 hr. (B) KIP2 is not regulated by p53. Blots containing total RNA prepared from A1–5 cells plated at 39.5°C or 32.5°C for 48 hr were probed with mouse CIP1 or KIP2 probes. The exposure time was 15 hr for CIP1 and 48 hr for KIP2.

there is a minor 1.4-kb species in most human tissues. In addition, there are a number of alternatively sized mRNA species apparent in mouse heart and testis, and human skeletal muscle. The 6-kb mRNA detected in mouse heart is not observed when a probe is used that contains only the kinase-inhibitory domain.

p57^{KIP2} expression during mouse development partially overlaps with *p21^{CIP1}*

The ability of *p57^{KIP2}* to function as a cell cycle inhibitor suggests that it might also function to mediate cell cycle arrest during development. Knowledge of the specific timing and location of *p57^{KIP2}* embryonic expression in vivo could provide evidence that this CKI is involved in terminal differentiation in a developing organism. The in vivo expression pattern of mouse *p57^{KIP2}* mRNA was examined during embryogenesis and in adult tissues by in situ hybridization. Embryos from day 9.5 postcoitum (pc) through day 15.5 pc were examined. *p57^{KIP2}* was expressed widely in brain, lens epithelium of the eye, skeletal muscle, and cartilage at all ages examined. A transverse section through the head of a 10-day pc mouse embryo showed expression of *p57^{KIP2}* in the neural epithelium, Rathke's pouch, and the otocyst (Fig. 5A). In a more caudal region, mRNA expression was observed in the dermamyotome where the first determined myocytes are localized (Fig. 5B), based on coexpression of myogenin in adjacent sections (data not shown). High level expression of *p57^{KIP2}* was seen later in development in skeletal muscle and cartilage of the cervical region (Fig. 5D) and developing forelimb (Fig. 5E) of 13.5- to 14.5-day pc embryos. Also at this stage in development

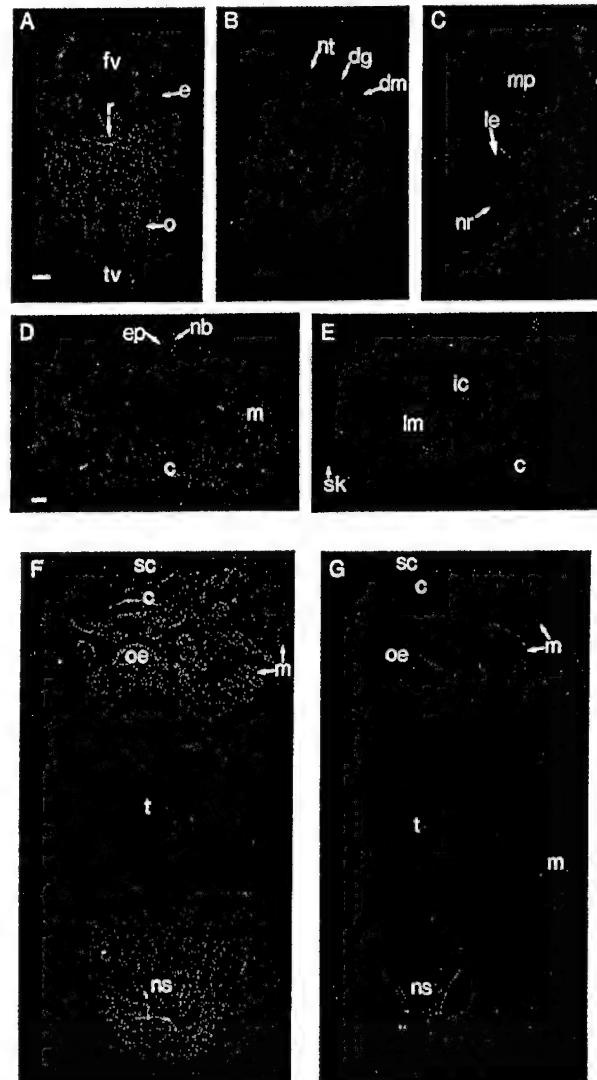


Figure 5. Expression of *p57^{KIP2}* during mouse embryogenesis. In situ hybridization was performed on specimens as described in Materials and methods. Antisense KIP2 RNA was used as probe for A–F whereas *p21^{CIP1}* was used as probe for G. (A) Transverse section through the head of a 10-day pc embryo. (B) Transverse section through the tail area of a 10-day pc embryo. (C) Coronal section through the head of a 13.5-day pc embryo. (D) Transverse section through the cervical region of a 14.5-day pc embryo. (E) Transverse section through the forelimb of a 14.5-day pc embryo. (F) Coronal section through the head and neck of a 14.5-day pc embryo. (G) Coronal section through the head and neck of a 14.5-day pc embryo probed for *p21^{CIP1}* (Parker et al. 1995). Abbreviations are (c) cartilage; (dg) dorsal root ganglion; (dm) dermamyotome; (e) eye; (ep) ependymal layer; (fv) fourth ventricle; (dg) dorsal root ganglion; (dm) dermamyotome; (le) lens epithelium; (lm) limb muscle; (m) muscle; (mp) maxillary process; (nb) migrating neuroblasts; (nr) neural retina; (ns) nasal septum; (nt) neural tube; (o) otocyst; (oe) oesophagus; (r) Rathke's pouch; (sk) skin; (sc) spinal cord; (t) tongue; (tv) third ventricle. The scale bar in A is 100 μ m and represents A and E–G. The scale bar in D is 100 μ m and represents B–D.

the lens epithelium showed intense expression of p57^{KIP2} (Fig. 5C). In general, p57^{KIP2} expression correlates with cells that have left the cell cycle.

The expression pattern for p21^{CIP1} has been established during embryogenesis and in adult tissues (Parker et al. 1995). It shows specific expression in a number of tissues during development including muscle, epidermis, certain epithelial cells, and cartilage, among others. For comparison, adjacent sections were probed for p21^{CIP1} and p57^{KIP2}. As seen in Figure 5, F–G, p57^{KIP2} and p21^{CIP1} coexpress in skeletal muscle, cartilage, and tongue muscle, although p57^{KIP2} seems to be expressed more heavily in these tissues. Differences in expression patterns of p57^{KIP2} and p21^{CIP1} can be observed in the nasal region (Fig. 5F–G) and in the skin of the developing forelimb where p57^{KIP2} is not expressed (Fig. 5E) in contrast to p21^{CIP1} expression in the outer layers of embryonic epidermis (Parker et al. 1995; data not shown). Owing to the abundance of muscle and cartilage tissue in an animal, the coexpression in these lineages gives the incorrect impression that p57^{KIP2} and p21^{CIP1} are generally coexpressed. However, during development, most sites of expression including the brain, epidermis, hair follicles, nasal epithelium, and eyes are nonoverlapping.

A survey of p57^{KIP2} expression by *in situ* hybridization in adult tissues revealed that it is expressed widely in brain, kidney, heart, lung, liver, and skeletal muscle (Fig.

6A,B,E–H). Expression is limited to the muscularis mucosa layer of the stomach and is completely absent in the small intestine (Fig. 6C,D). In contrast, p21^{CIP1} is expressed heavily in the intestinal villi of the small intestine and in the surface epithelium of the stomach (Parker et al. 1995) but not in the muscularis mucosa layer of the stomach.

KIP2 localizes to chromosome 11p15.5, a locus implicated in several human cancers

The chromosomal localization of the KIP2 gene was identified by fluorescent *in situ* hybridization (FISH) (Ijdo et al. 1992). It localized to the telomeric end of human chromosome 11 at 11p15.5 (Fig. 7), a position implicated in a number of human cancers (see Discussion). Thus, the CKI p57^{KIP2} is a candidate tumor suppressor gene, consistent with a role in maintenance of the nonproliferative state in adult tissues.

Discussion

We have employed a genetic approach to identify genes encoding proteins that physically associate with cyclins and cyclin-dependent kinases. Using a modified version of the two-hybrid system, we identified KIP2, which en-

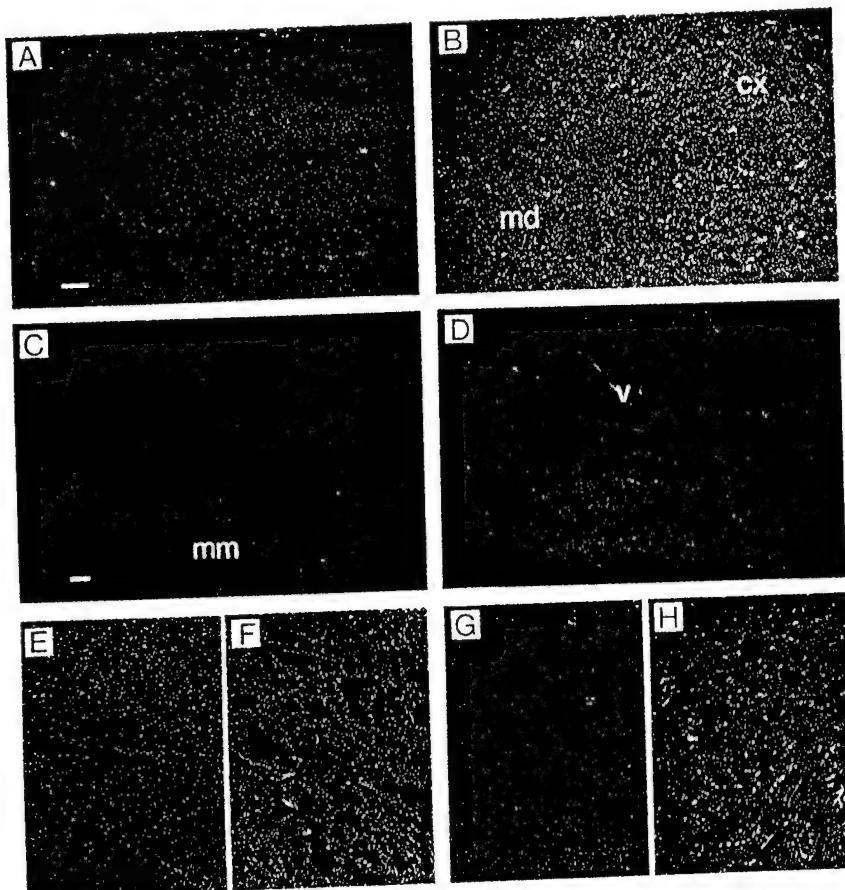


Figure 6. Expression of p57^{KIP2} in adult mouse tissues by *in situ* hybridization. Sections of brain (A); kidney (B); stomach (C); small intestine (D); heart (E); lung (F); liver (G); and skeletal muscle (H) from a 4-month-old C57B mouse were probed for KIP2 mRNA as described in Materials and methods. Abbreviations are (cx) renal cortex; (md) renal medulla; (mm) muscularis mucosae; and (v) intestinal villi. The scale bar in A is 100 μ m and represents A, B, and D–H. The scale bar in C is 100 μ m.

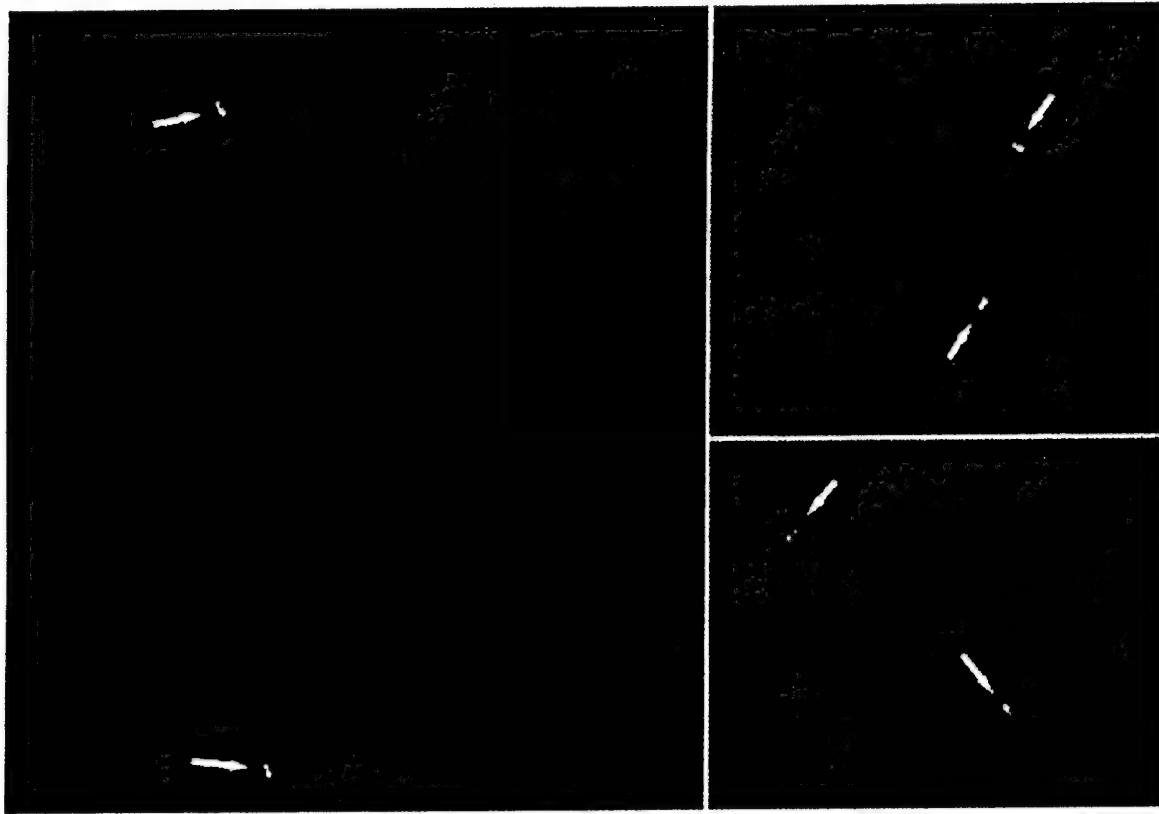


Figure 7. The human KIP2 gene is located on chromosome 11p15.5. *In situ* hybridization was performed on mitotic chromosomes using fluorescently labeled KIP2 DNA. Three independent spreads of metaphase chromosomes are shown. Arrows indicate the localization of KIP2 on the telomeric end of chromosome 11 at 11p15.5.

codes a novel inhibitor of cyclin-dependent kinases. KIP2 encodes a 335 amino acid protein in mouse and a 316 amino acid protein in humans, both of which migrate as 57 kD by SDS-PAGE, very close to cyclin A at 58 kD. Comigration with cyclin A may have prevented earlier biochemical detection. KIP2 shows specificity for G₁ Cdk's. It can bind several Cdk's in a cyclin-dependent manner, including cyclin A/Cdk2, cyclin E/Cdk2, cyclin E/Cdk3, cyclin D2/Cdk4, and, to a lesser extent, cyclin D2/Cdk6. Furthermore, p57^{KIP2} can inhibit the kinase activity of the G₁ cyclin Cdk2, Cdk3, and Cdk4 complexes. Thus, KIP2 is capable of inhibiting several cyclin-dependent kinases with demonstrated roles in the G₁/S-phase transition. This places KIP2 in a position to play an important regulatory role in control of cell proliferation.

The cyclin dependence of binding and inhibition of p57^{KIP2} is similar to that observed for p21^{CIP1} and p27^{KIP1} (Harper et al. 1995) and suggests that p57^{KIP2} can also act as a buffer to titrate cyclin/Cdk complexes as they are formed. One notable difference is the reduced activity toward cyclin D2/Cdk6 complexes that are efficiently inhibited by p21^{CIP1} (Harper et al. 1995). This difference may indicate a potential mechanism of p57^{KIP2} resistance, that is, dependence on Cdk6 instead of Cdk4 for cyclin D kinase activity. Overproduction of

p57^{KIP2} can efficiently arrest SAOS2 cells in the G₁ phase of the cell cycle, consistent with its specificity for inhibition of G₁ cyclin/Cdk complexes. Furthermore, it can be found associated with Cdk2 in these transfected cells indicating that p57^{KIP2} is likely to mediate cell cycle arrest through its known biochemical activity of binding and inhibiting Cdk's *in vivo*.

p57^{KIP2} is the most structurally diverse member of the p21^{CIP1} family of CKIs, consisting of four distinct regions. It is more closely related to p27^{KIP1} in the kinase inhibitory domain. In addition, it shares strong similarity with p27^{KIP1} in the carboxy-terminal region termed the QT box. The relatedness suggests that the QT box has a defined function, most likely in protein-protein interaction. Because p21^{CIP1} appears to have a dual inhibitory function *in vitro*, it is tempting to speculate that the other members of this class will also have dual functions. However, a cytomegalovirus (CMV) expression construct expressing domains II-IV of p57^{KIP2} or the carboxy-terminal domain of p21^{CIP1} had no effect on cell cycle distribution of SAOS2 cells (C. Bai, J.W. Harper, and S.J. Elledge, unpubl.). Thus, a secondary inhibitory function for either of these CKIs has not been demonstrated *in vivo*.

The most striking features of the primary structure of KIP2 are the internal repeat domains. Mouse p57^{KIP2} has

a proline-rich region and an acidic repeat region. The human cDNA that we have isolated has a proline-alanine repeat region termed the PAPA repeat. A number of issues concerning these motifs remain unanswered. For example, it is not known definitively whether the mouse and human genes are true homologs or members of a related family. The sequence conservation in the Cdk inhibitory and QT box domains suggests that they are closely related. The absence of sequence conservation in the internal region could be attributable to the lack of functional conservation. It is possible that the internal domains are merely spacer regions needed to appropriately separate the amino- and carboxy-terminal domains, and their length, not their composition, is the critically conserved feature. Alternatively these sequences may serve as important sites of protein-protein interaction. One possible function for domains II-IV is to recruit substrates to the Cdk/cyclin complexes. It has been shown recently that multiple molecules of p21^{CIP1} are necessary to inhibit Cdk5 (Zhang et al. 1994; Harper et al. 1995). Thus, these inhibitors can be part of active complexes and any additional proteins recruited into these complexes are potential substrates of these Cdk5. These multidomain inhibitors could serve as adapters that allow signals to be generated from Cdk5 in a cell during the process of terminal differentiation.

p57^{KIP2} is expressed in several embryonic and adult tissues derived from the ectoderm, mesoderm, and endoderm. Overall there is a strong correlation between arrest of cell proliferation and p57^{KIP2} expression, strongly suggesting that expression of p57^{KIP2} and other cyclin kinase inhibitors will be coupled to activation of cell differentiation in multiple cell lineages. In muscle it has been shown that p21^{CIP1} is also induced during terminal differentiation indicating a potentially redundant role with p57^{KIP2} in this tissue. It is likely that a number of redundant mechanisms will cooperate to ensure permanent exit from the cell cycle in terminally differentiated tissues. Although there may be some redundancy with p21^{CIP1}, there are also complementary patterns of expression suggesting unique functions for each protein. In adults, p57^{KIP2} mRNA is found in a wide range of tissues, indicating a role in maintenance of the nonproliferative state throughout life.

The chromosomal location of human KIP2, 11p15.5, marks it as a candidate tumor suppressor gene. This region has been investigated intensively because of frequent loss of heterozygosity at this locus in a number of human cancers including breast cancer, bladder, lung, ovarian, kidney, and testicular carcinomas (for review, see Seizinger et al. 1991). Several types of childhood tumors, including Wilms' tumor, adrenocortical carcinoma, rhabdomyosarcoma, and hepatocellular carcinoma, display a specific loss of maternal 11p15 alleles, suggesting a role for genomic imprinting. Chromosome transfer experiments have provided evidence that a tumor suppressor gene resides at this locus, the WT2 gene involved in Wilms' tumor and possibly rhabdomyosarcoma (for review, see Hastie 1994), either of which could be attributable to loss of a Cdk inhibitor. In addition,

rearrangements in the 11p15 region have been linked to Beckwith-Wiedemann Syndrome (BWS), which is characterized by numerous growth abnormalities, including macroglossia (enlarged tongue), gigantism, visceromegaly (enlarged organs), exomphalos (umbilical protrusion), and an increased risk (7.5%) of childhood tumors (Weidemann 1983). The BWS occurs with an incidence of 1 in 13,700 births, 85% of which are sporadic and 15% familial (Pettenucci et al. 1986). Genetic analysis indicates maternal carriers, also suggesting a role for genomic imprinting (for review, see Junien 1992). Several features of the KIP2 gene make it a reasonable candidate for a mediator of some of the phenotypes associated with BWS. First, a Cdk inhibitor could potentially explain both overgrowth and tumorigenesis phenotypes. Furthermore, the expression pattern of KIP2 in mouse correlates with areas known to be affected in BWS, including the tongue, kidney, and muscle. Finally, the presence of the PAPA repeat region might provide a high frequency mechanism for expansion mutation akin to the triplet repeat mechanism known to operate in fragile X syndrome and other diseases. Genetic analysis of alterations in the KIP2 gene in tumors or affected individuals will be necessary to determine which, if any, of these 11p15-associated diseases are attributable to p57^{KIP2}.

Materials and methods

Isolation of p57^{KIP2} cDNAs

Two-hybrid screens were performed using pAS1-cyclin D1 in yeast strain Y190 (Durfee et al. 1993; Harper et al. 1993) and a mouse day-10.5 pc embryonic cDNA library fused with the GAL4 activation domain. Plasmids were recovered into *Escherichia coli* strain JA226 and reintroduced into Y190 strains containing either pAS1-cyclin D1 or other pAS2 plasmids expressing various GAL4 DNA-binding domain fusion proteins to test for specificity of the interaction with cyclin D1. Clones selective for interaction with cyclin D1 were subjected to DNA sequence analysis (Sequenase, U.S. Biochemical) using either library plasmids or pBluescript subclones.

A human homolog of mouse p57^{KIP2} was isolated by low stringency hybridization (Elledge and Davis 1990) using a embryonic library in λ gt10 and a probe containing nucleotides 1-581 of the mouse cDNA. Phage inserts were subcloned into pBluescript for sequence analysis.

Protein expression and purification

For expression of mouse p57^{KIP2} as a glutathione S-transferase (GST) fusion protein, the open reading frame was engineered to contain an NcoI site at the initiation codon (MERL), cloned into a modified version of pGEX-2TK, and purification was accomplished as described previously for GST-p21 (Harper et al. 1993). The concentration of GST-p57 was determined by Bradford analysis. p57^{KIP2} in vitro translation was accomplished using TnT reticulocyte system (Promega) in conjunction with pBluescript-p57. For expression of p57^{KIP2} in SF9 cells, the KIP2 open reading frame was cloned into pBlueBacHis (Invitrogen) and recombinant baculovirus generated using Baculogold (Pharminogen) as described by the manufacturer. This virus produces a p57 protein with a 41 amino acid amino-terminal extension containing a polyhistidine tag and sequences from T7 gene 10,

which are recognized by anti-T7 Tag antibodies (Novagen). [³⁵S]Methionine-labeled sf9 extracts and purified cyclin/Cdk complexes were generated 44 hr postinfection as described previously (Matsushime et al. 1992; Harper et al. 1993, 1995). GST-Rb and histone H1 kinase assays were performed as described (Harper et al. 1993).

Antibody production

Polyclonal antibodies against mouse p57^{KIP2} were generated in rabbits using GST-p57ΔC (residues 1–167) purified by preparative SDS-PAGE. Antibodies for immunoprecipitation were depleted of GST antibodies using GST protein covalently linked to glutathione-Sepharose followed by affinity purification using GST-p57 linked to glutathione-Sepharose. Proteins were coupled to Sepharose using dimethyl palmitate (Harlow and Lane 1988). Antibodies for immunoblotting were purified by first depleting antisera of GST reactive antibodies and then affinity purifying p57-specific antibodies using GST-p57 immobilized on nitrocellulose. Antibody concentrations were determined by Bradford analysis.

In vitro binding

To examine the interaction of p57^{KIP2} with Cdks, 10 µl of [³⁵S]methionine-labeled sf9 cell lysate containing the indicated proteins were mixed with 4 µl of in vitro translated p57. After 10 min on ice, extracts were diluted with 150 µl of binding buffer (40 mM Tris-HCl, 150 mM NaCl, 0.5% NP-40, 10 mM NaF, 2 mM EDTA, 5 µg/ml of antipain, leupeptin, and aprotinin) and Cdks then were immunoprecipitated using 10 µl of protein A-Sepharose (Pharmacia) and 0.5–2 µg of the appropriate antibody. Immune complexes were washed three times with 1 ml of binding buffer prior to SDS-PAGE and autoradiography. To examine the interaction of p57 with cyclins, [³⁵S]methionine-labeled sf9 lysates for cyclins A, D1, and D2, and T7Tag-p57 were first depleted of Cdk-associated cyclins using 25 µl of p13-agarose (Oncogene Science). The indicated extracts were mixed and immunoprecipitated with either antibodies directed against the cyclin or anti-T7 Tag prior to SDS-PAGE and autoradiography. Cyclin A antibodies were from a previous study (Elledge et al. 1992), whereas anti-cyclin D1 and D2 were from Santa Cruz Biocytomics.

To examine the association of p57^{KIP2} with Cdk2 in transfected SAOS-2 cells, extracts were generated from cells transfected with either pCMV-p57 or pCMV, a negative control, as described below. Cells from two 10 cm dishes were lysed in binding buffer. Extracts (0.4 mg of protein) were immunoprecipitated with 0.3 µg of either affinity purified anti-p57 or normal rabbit sera. Immune complexes washed three times with binding buffer and immune complexes separated by SDS-PAGE prior to immunblotted with anti-Cdk2 (Santa Cruz). Detection was accomplished using ECL (Amersham).

Transfections

SAOS-2 cells were maintained in Dulbecco's modified Eagle medium containing 10% fetal bovine serum at 37°C in 5% CO₂. Transient transfections for flow cytometry were performed using the calcium phosphate method (van der Heuvel and Harlow 1993; Zhu et al. 1993). Briefly, the indicated plasmids (20 µg) were cotransfected with pCMV-CD20 (2 µg) into SAOS-2 cells (20% confluence). Forty-eight hours post-transfection, cells were harvested with phosphate-buffered saline (PBS) containing 0.1% EDTA prior to staining with fluorescein isothiocyanate-conjugated anti-CD20 antibodies (Becton-Dickinson). Cells

were washed with PBS, then fixed with 80% ethanol, and DNA stained with propidium iodide (10 µg/ml) containing 25 µg/ml of ribonuclease A prior to flow cytometry using a Becton Dickinson FACScan. DNA content in 4000–6000 CD20-positive cells is presented in the DNA histograms. For immunoblot analysis of transfected cells, 10 µg of whole-cell lysates was separated by SDS-PAGE, transferred to nitrocellulose, and probed with affinity-purified anti-p57.

p57^{KIP2} expression

Human and mouse tissue Northern blots (Clonetech) were probed at high stringency with full-length human or mouse cDNAs labeled with [α -³²P]dCTP. For in situ hybridization, embryos or tissues were collected at the indicated times, fixed in 3% paraformaldehyde, embedded in paraffin, and sectioned on a Microm microtome at 6 µm. Specimens were hybridized with riboprobes labeled with α -³⁵S-labeled UTP essentially as described previously (Lutz et al. 1994). pBluescript p57 was linearized with *Kpn*I or *Bam*HI, and sense and antisense transcripts generated using either T7 or T3 polymerase, respectively. Specimens were photographed by double exposure using dark-field illumination with a red filter and Hoechst epifluorescence optics.

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Cdk-interacting protein 1 directly binds with proliferating cell nuclear antigen and inhibits DNA replication catalyzed by the DNA polymerase δ holoenzyme

(cell cycle regulation/processivity/protein–protein interaction)

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ABSTRACT Cdk-interacting protein 1 (Cip1) is a p53-regulated 21-kDa protein that inhibits several members of the cyclin-dependent kinase (CDK) family. It was initially observed in complexes containing CDK4, cyclin D, and proliferating cell nuclear antigen (PCNA). PCNA, in conjunction with activator 1, acts as a processivity factor for eukaryotic DNA polymerase (pol) δ , and these three proteins constitute the pol δ holoenzyme. In this report, we demonstrate that Cip1 can also directly inhibit DNA synthesis *in vitro* by binding to PCNA. Cip1 efficiently inhibits simian virus 40 replication dependent upon pol α , activator 1, PCNA, and pol δ , and this inhibition can be overcome by additional PCNA. Simian virus 40 DNA replication, catalyzed solely by high levels of pol α -primase complex, is unaffected by Cip1. Using the surface plasmon resonance technique, a direct physical interaction of PCNA and Cip1 was detected. We have observed that Cip1 efficiently inhibits synthesis of long (7.2 kb) but not short (10 nt) templates, suggesting that its association with PCNA is likely to impair the processive movement of pol δ during DNA chain elongation, as opposed to blocking assembly of the pol δ holoenzyme. The implications of the Cip1-PCNA interaction with respect to regulation of DNA synthesis, cell cycle checkpoint control, and DNA repair are discussed.

The eukaryotic cell cycle is regulated by a family of cyclin-dependent kinases (CDKs). Several small proteins have been detected that bind and inhibit CDKs (reviewed in ref. 1); one such protein, cdk-interacting protein 1 (Cip1; also known as wild-type p53-activated fragment 1, or WAF-1), was initially observed in a quaternary complex that also included cyclin D, CDK4, and proliferating cell nuclear antigen (PCNA) (2). Cip1 can directly interact with CDKs (3–5), indicating that PCNA is not required for the interactions between Cip1 and CDKs.

Cip1 is regulated by the tumor suppressor gene p53, a transcription factor that controls the G₁ cell cycle arrest checkpoint in response to DNA damage (6). Cip1 is transcriptionally induced by DNA damage in a p53-dependent fashion and may mediate cell cycle arrest (7). The G₁ checkpoint is thought to arrest the cell cycle to prevent DNA replication of damaged templates while allowing repair.

Although kinases are thought to regulate DNA synthesis, the appearance of Cip1 and PCNA in a complex suggested the possibility of a more direct link to replication. PCNA, in conjunction with activator 1 (A1; also known as RF-C), acts as a processivity factor for DNA polymerase (pol) δ (8–10). Recent studies have shown that PCNA (as a trimer) and the

β subunit (a dimer) of DNA pol III holoenzyme of *Escherichia coli* both form a tight clamp that tethers their cognate DNA polymerase to the template and translocates along the DNA with the polymerase during replication (11–13). Thus any agent that can interact with the sliding clamp might impede the movement of the replication complex.

Here we demonstrate that Cip1 directly binds PCNA to form a complex that inhibits the activity of the PCNA-dependent pol δ holoenzyme, which reduces the replication of simian virus 40 (SV40) DNA and the elongation of primed DNA templates. However, SV40 DNA replication carried out with the pol δ -independent monopolymerase system was unaffected by Cip1. The inhibitory effects of Cip1 were independent of the cdk2–cyclin A complex.

MATERIALS AND METHODS

Preparation of DNAs and Proteins. (dT)_{12–18} and (dA)₄₅₀₀ were obtained from Life Sciences Inc.; poly(dA) was annealed to oligo(dT) (20:1) as described (14). Singly primed DNA was prepared by hybridizing a 2-fold molar excess of a 34-nt oligonucleotide to circular single-stranded M13 mp7 (7.2 kb) DNA (containing 10–15% linear molecules) at nucleotide positions 6300–6633. The annealed product was labeled by the incorporation of a single dCMP residue (corresponding to position 6299) using the Klenow fragment of DNA pol I. The resulting product (\approx 2000 cpm/fmol), after phenol extraction, was isolated by filtration through a G-50 Sephadex column. Cytosolic extracts of HeLa cells, SV40 origin-containing DNA (pSV01ΔEP), human single-stranded DNA-binding protein (HSSB; 600 units/mg of protein; also called RP-A), pol α -primase complex (3.7 \times 10³ and 4.9 \times 10³ units/mg, respectively), SV40 large tumor antigen (T antigen), and PCNA (2000 units/mg of protein) were prepared as described (15–17). pol δ , isolated from HeLa cells, was purified as described by Lee *et al.* (14). The preparation used here contained 1500 units/mg of protein. The purification of A1, based on its ability to stimulate pol δ activity, was carried through procedure 2, as described (14). In all experiments reported here, the single-stranded DNA-cellulose fraction (1200 units/mg of protein) was used. Cip1 was expressed in *E. coli* and isolated as described (3). Cip1 (at concentrations between 0.5 and 1 mg/ml) was stored at a salt concentration of 0.2–0.4 M NaCl to avoid aggregation and was diluted 5- to 10-fold prior to use. Human cyclin A and cdk2 (kindly

Abbreviations: HSSB, human single-stranded DNA binding protein; SSB, single-stranded DNA binding protein; A1, activator 1; PCNA, proliferating cell nuclear antigen; pol, DNA polymerase; SV40, simian virus 40; T antigen, SV40 large tumor antigen; Cip1, p21 cdk-interacting protein 1; CDK, cyclin-dependent kinase; RU, response units.

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provided by E. Gibbs, Memorial Sloan-Kettering Cancer Center), overexpressed in *E. coli*, were prepared as described (18). The β and δ subunits of the *E. coli* pol III holoenzyme were both overexpressed and cloned in *E. coli* and prepared as described (19, 20).

Binding and Replication Assays. The immobilization of PCNA and the *E. coli* β subunit to sensor chips was carried out using the carbodiimide covalent linkage procedure following the manufacturer's (Pharmacia Biosensor) instructions. The response units (RU) indicated the concentration of PCNA and β subunit covalently linked to the chip and are reported in the figure legend.

SV40 DNA replication, the elongation of poly(dA)-oligo(dT), and singly primed M13 DNA were carried out as described (14). Where indicated, elongation reactions were analyzed by alkaline agarose gel electrophoresis as detailed in the figure legends.

RESULTS

Influence of Cip1 on SV40 DNA Replication by Crude Extracts and the Monopolymerase System. The influence of Cip1 on the T antigen-dependent replication of SV40 DNA using crude extracts of HeLa cells was examined. In this reaction, DNA synthesis is dependent on the combined action of the pol α -primase complex for the initiation of DNA chains and the pol δ holoenzyme for their elongation. As shown in Fig. 1, DNA synthesis, measured by incorporation of labeled deoxynucleotides, was inhibited by increasing levels of Cip1. In contrast, SV40 DNA synthesis carried out by the monopolymerase system was not affected by Cip1. In the monopolymerase system, relatively high levels of pol α carry out both leading- and lagging-strand synthesis, obviat-

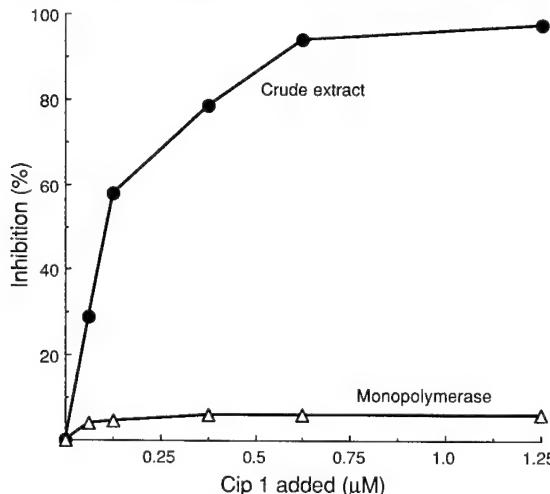


FIG. 1. Influence of Cip1 on SV40 DNA replication by crude extracts of HeLa cells and by the monopolymerase system. Replication of SV40 DNA by crude extracts was carried out in reaction mixtures (40 μ l) containing 0.3 μ g of pSV01 Δ EP, 0.83 μ g of SV40 T antigen, 0.3 μ g of HSSB, 186 μ g of crude extract of HeLa cells, Cip1 as indicated, and other reagents as described (14, 17). Reaction mixtures were incubated for 90 min at 37°C, and the amount of acid-insoluble radioactivity formed (using [α -³²P]dCTP) was measured. In the absence of Cip1, 74.4 pmol of dCMP was incorporated. In the monopolymerase reactions, reaction mixtures (40 μ l) contained the same amount of DNA, SV40 T antigen, and HSSB as above, but in place of the crude extract of HeLa cells, purified pol α -primase complex (0.4 and 1.1 units, respectively), 12 ng of *E. coli* cloned vaccinia topoisomerase I (a gift from S. Shuman, Memorial Sloan-Kettering Cancer Center), and 3 μ g of bovine serum albumin were added. Cip1 was added as indicated and other reagents were added as described (14). In the absence of Cip1, 28.2 pmol of dCMP was incorporated into DNA after 90 min at 37°C.

ing the requirement for the PCNA-dependent pol δ holoenzyme (16). Since SV40 DNA replication with crude extracts and the monopolymerase system depend upon SV40 T antigen, HSSB, and the pol α -primase complex for activity, these observations suggest that Cip1 blocked SV40 replication in crude extracts by inhibiting the pol δ holoenzyme system.

The inhibition of replication with crude extracts by Cip1 could be partially reversed by supplementing reactions with purified PCNA. Thus, in the presence of 50 and 100 ng (120 nM) of Cip1, which inhibited replication 38% and 65%, respectively, the addition of 100 ng (86 nM monomer) of PCNA reduced the inhibition to 14% and 45%, respectively (data not presented). The addition of this level of PCNA had virtually no effect on DNA synthesis in the absence of Cip1. This suggests that the inhibition by Cip1 in this system is specifically due to an effect on the action of PCNA.

Influence of Cip1 on the pol δ Holoenzyme-Catalyzed Elongation of Primed DNA Templates. A direct measurement of the activity of the pol δ holoenzyme, which consists of PCNA, A1, and pol δ , and of the influence of Cip1 was carried out using primed DNA templates. For this purpose, (dA)₄₅₀₀-(dT)₁₂₋₁₈ was used as the primer template. The synthesis of poly(dT) with this substrate is dependent on ATP, a SSB, PCNA, A1, pol δ , and dTTP. As shown in Fig. 2, elongation of oligo(dT) was markedly inhibited by Cip1, and the extent of inhibition depended on the level of PCNA added. In the presence of 10 ng (11.4 nM monomer) of PCNA, 50% inhibition of poly(dT) synthesis was observed in the presence of 50 ng (80 nM) of Cip1. Higher levels of PCNA require higher Cip1 levels to inhibit the reaction. Under the conditions of the experiment, the inhibitory effects of Cip1 were unaffected by increasing the levels of A1, pol δ , HSSB, or addition of cdk2 in the presence or absence of cyclin A (data not presented). These observations suggest that Cip1 inhibits the activity of PCNA independent of the presence of cdk2-cyclin A.

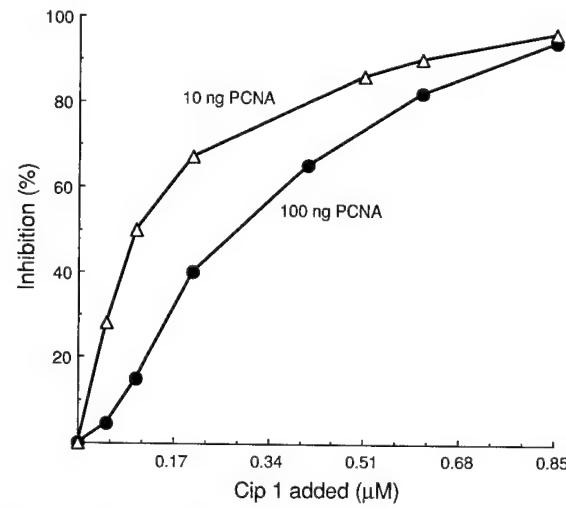


FIG. 2. Influence of Cip1 on the synthesis of poly(dT) by the pol δ holoenzyme. Reaction mixtures (30 μ l) contained 10 mM Tris-HCl (pH 7.8), 10 mM creatine phosphate (di-Tris salt, pH 7.7), 1 μ g of creatine phosphokinase, 2 mM dithiothreitol, bovine serum albumin (150 μ g/ml), 30 μ M [α -³²P]dTTP (1200 cpm/pmol), 2 mM ATP, 7 mM MgCl₂, 300 pmol of (dA)₄₅₀₀-(dT)₁₂₋₁₈ (20:1), 0.6 μ g of HSSB, 0.2 unit of A1, 0.1 unit of pol δ , PCNA, and Cip1 as indicated. After 20 min at 37°C, the amount of acid-insoluble poly(dT) formed was measured. In these reactions, A1 and pol δ were added after the addition of all other reagents, which were assembled at 0°C. In the absence of Cip1, dTTP incorporation with 100 and 10 ng of PCNA was 132 and 55.2 pmol, respectively. In the absence of PCNA, A1, or pol δ , dTTP incorporation was 1.80, 1.46, and <0.1 pmol, respectively.

If the loading of PCNA onto primer ends was completely blocked by Cip1, the pol δ -catalyzed elongation reaction would be totally inhibited. Such a mechanism would result in an all-or-none synthesis of DNA chains. In reactions partially inhibited by Cip1, the size of extension products would be unaffected, but their yield would be reduced. To determine whether Cip1 blocked the elongation of primed templates in an all-or-none manner, the elongation of a single labeled primer annealed to single-stranded circular M13 DNA was carried out. In these experiments, two different amounts of PCNA, 50 ng (84 nM monomer) and 100 ng were used, each in the presence of 50 ng (120 nM) and 100 ng of Cip1. Under these conditions, nucleotide incorporation was inhibited between 30% and 80% (data not presented). Analysis of the replication products by alkaline agarose gel electrophoresis indicated that Cip1 caused a marked reduction in the formation of longer products, concomitant with an increased accumulation of shorter chains (Fig. 3, lanes 5–8). Again, the addition of cdk2 (450 nM) and cyclin A (260 nM) did not alter the quantitative distribution of products formed in the presence (lanes 7–10) or absence (lanes 3, 4, 11, and 12) of Cip1.

These results indicate that Cip1 inhibition does not lead to an all-or-none effect on the pol δ -catalyzed elongation reaction and most likely interferes with the sliding of PCNA along the DNA or its association with pol δ .

Further support for this notion was obtained by examining the influence of Cip1 on short-chain elongation of the singly primed M13 DNA containing a labeled primer. In these experiments, chains were elongated for only 10 nt by the inclusion of ddCTP as a chain terminator (Fig. 4). In the

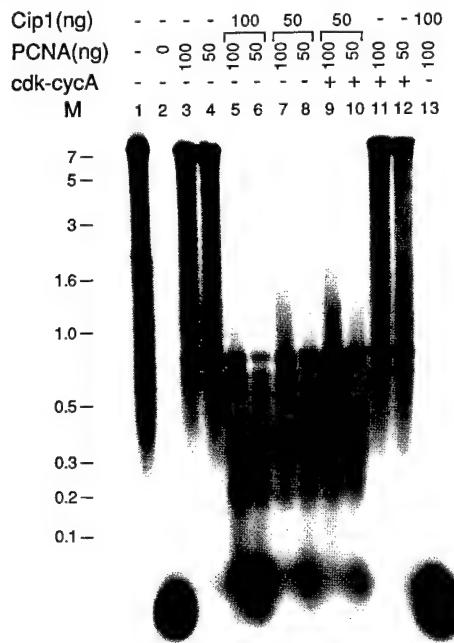


Fig. 3. Influence of Cip1 on the elongation of labeled singly primed DNA by pol δ holoenzyme. Reaction mixtures (20 μ l) contained 30 mM Tris-HCl (pH 7.8), 2 mM dithiothreitol, bovine serum albumin at 150 μ g/ml, 100 μ M dNTPs, 2 mM ATP, 7 mM MgCl₂, 16.2 fmol of singly primed M13 mp7 single-stranded circular DNA, and the indicated amounts of Cip1 were preincubated at 37°C for 10 min. HSSB (210 ng), pol δ (0.025 unit), A1 (0.05 unit), and PCNA (2 ng, 22 fmol of trimer) were added, and primer extension was carried out at 37°C for 8 min. Reactions were terminated by the addition of 3 μ l of 0.1 M EDTA/2% SDS, boiled for 5 min to dissociate the labeled oligonucleotide products from the M13 circular templates, and electrophoresed through a 12% polyacrylamide gel containing TBE buffer.

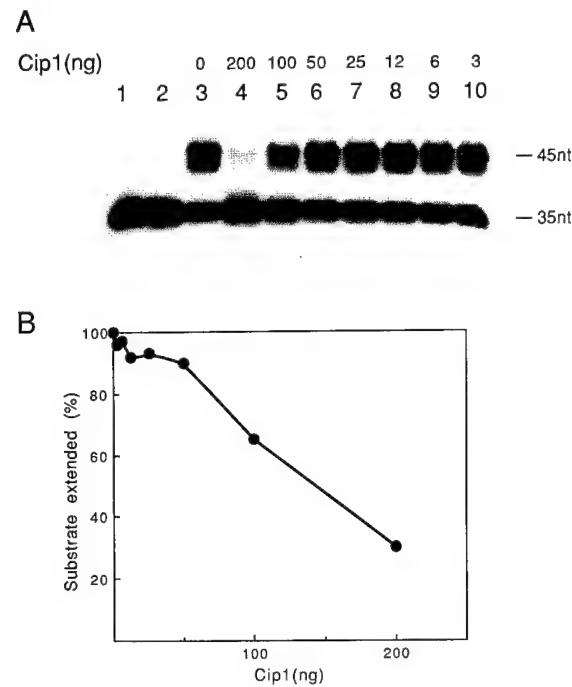


Fig. 4. Influence of Cip1 on the synthesis of a 10-nt DNA fragment by the pol δ holoenzyme. Reaction mixtures (20 μ l) containing 60 mM Tris-HCl (pH 8.0), 60 mM creatine phosphate, 10 mM MgCl₂, 1 mM dithiothreitol, 5 μ g of bovine serum albumin, 3 mM ATP, 100 mM each of dATP, dGTP, dTTP, and ddCTP, 5 fmol of labeled, singly primed M13 mp7 single-stranded circular DNA, and the indicated amounts of Cip1 were preincubated at 37°C for 10 min. HSSB (210 ng), pol δ (0.025 unit), A1 (0.05 unit), and PCNA (2 ng, 22 fmol of trimer) were added, and primer extension was carried out at 37°C for 8 min. Reactions were terminated by the addition of 3 μ l of 0.1 M EDTA/2% SDS, boiled for 5 min to dissociate the labeled oligonucleotide products from the M13 circular templates, and electrophoresed through a 12% polyacrylamide gel containing TBE buffer. (A) Autoradiograph of the dried gel. Lane 1, enzymes omitted; lane 2, no PCNA; lane 3, complete reaction but lacking Cip1. (B) Quantitation of the Cip1 inhibition of primer extension. Extension of 2.7 fmol of substrate corresponded to 100% activity.

presence of 0.25 nM template and 2 ng of PCNA (3.3 nM monomer), short-chain elongation was reduced <10% by concentrations of Cip1 up to 50 ng (120 nM). At higher levels of Cip1 (480 nM), this reaction was inhibited up to 70%. This inhibition was probably due to impaired loading of PCNA on the primer end; when PCNA was first loaded onto the primer end in the presence of A1 and the complex was then treated with 200 ng of Cip1 (480 nM), the short-chain elongation reaction was reduced by only 10% (data not shown).

We interpret the short-chain elongation results as follows. In the presence of levels of Cip1 that inhibit extensive elongation, there is no inhibition of short-chain elongation because the enzyme translocates over a distance that is not long enough to reflect its impaired movement. The likely mechanism by which Cip1 interferes, in this short chain reaction, is by blocking the loading of the PCNA clamp on the primer end.

Direct Interaction Between Cip1 and PCNA. Direct evidence that Cip1 physically interacts with PCNA was obtained using the surface plasmon resonance technique (21). For the experiment shown in Fig. 5, human PCNA (expressed in *E. coli*) was coupled to the sensor surface over which a solution containing Cip1 was passed. An immediate burst in accumulation of mass was observed upon exposure to Cip1, plateauing at a stoichiometry of \approx 2.3 molecules of Cip1 (as monomer) per molecule of PCNA trimer (Fig. 5A). After 7 min, equilibration buffer lacking Cip1 was passed over the surface,

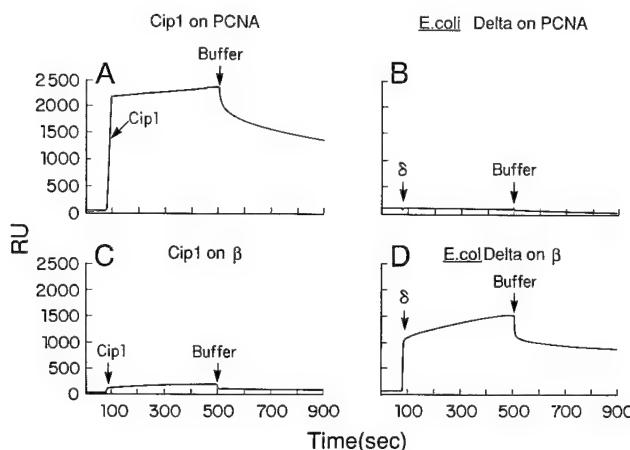


FIG. 5. Analysis of direct Cip1-PCNA interaction by SPR. (A) PCNA (4900 RU) was attached to a sensor chip by carbodiimide coupling. Assuming 1000 RU is equivalent to 10 mg/ml and a volume of 120 pL on the chip surface, the chip contained 67 fmol of PCNA (as trimer). During a 7-min time interval, 0.14 nmol of Cip1 (as monomer) was passed over the chip in a volume of 35 μ L of 10 mM Hepes-HCl (pH 7.4), 150 mM NaCl, 3.4 mM EDTA, and 1% glycerol, followed by a wash using the same buffer but lacking Cip1. (B) During a 7-min period, 0.13 nmol of *E. coli* δ subunit was passed over the PCNA chip followed by a wash as in A. (C) *E. coli* β subunit (3600 RU; 53 fmol as dimer in 120 pL) was linked to a sensor chip by carbodiimide coupling, and then Cip1 was passed over the chip, followed by a wash lacking Cip1 as described in A. (D) *E. coli* δ subunit was passed over the β chip followed by a wash as described in A.

resulting in the dissociation of Cip1, which was detected by a loss in mass that followed a first-order decay ($k_{off} = 6.2 \times 10^{-4} \text{ s}^{-1}$). To demonstrate that PCNA is specific in binding Cip1, the δ subunit of *E. coli* pol III holoenzyme was passed over PCNA, and no interaction was observed (Fig. 5B). The sliding clamp analogous to PCNA in the *E. coli* system is the β subunit, which tethers the pol III holoenzyme to DNA (summarized in ref. 22). When the β subunit was attached to a sensor chip and then Cip1 was passed over it, no significant interaction between β and Cip1 was detected, which indicates that Cip1 interacts specifically with PCNA (Fig. 5C). The β subunit binds directly to the δ subunit of the pol III holoenzyme (22). To show that the immobilized β subunit was active in protein binding, the δ subunit was passed over β (Fig. 5D). As shown, a strong signal was observed that plateaued at a stoichiometry of 0.85 molecule of δ (as monomer) per molecule of β (as dimer), consistent with the $\beta_2\delta_1$ stoichiometry measured by other techniques (V. Naktinis and M.O., unpublished observations).

The interaction between Cip1 and PCNA was further confirmed by analysis of the complex by gel filtration. The Cip1-PCNA polypeptides comigrated and shifted the elution volume of the PCNA trimer (molecular mass = 87 kDa) to a larger Stokes' radius that corresponded to a mass of 120–150 kDa (data not presented).

DISCUSSION

The results presented here demonstrate that Cip1 directly interacts with PCNA, forming a complex that can be readily detected by real-time interaction analysis using the BIACore (Pharmacia Biosensor). The strong RU observed revealed that each mol of PCNA trimer complexed 2.3 mol of Cip1, almost equivalent to the binding of one Cip1 molecule to one PCNA molecule. The lower observed value may represent the unavailability of portions of the PCNA trimer covalently linked to the sensor chip. Analysis of Cip1 interaction with *E. coli* pol β subunit revealed specificity for PCNA. Further

direct evidence for the interaction between Cip1 and PCNA was obtained by gel-filtration analysis.

The complex formed between PCNA and Cip1 reduced the ability of PCNA to support the pol δ -catalyzed elongation reactions. At molar ratios of Cip1 to PCNA trimer between 2 and 5, the elongation of singly primed M13 templates was inhibited, resulting in a marked decrease in the synthesis of full-length products and the accumulation of DNA chains 0.1–1 kb in length. At higher molar ratios, the accumulation of short DNA chains was reduced.

The steps involved in formation of the PCNA clamp and its loading by A1 on primed DNA templates in the eukaryotic system (pol δ and pol ϵ) have been elucidated (8–11). The initial step in this process involves the selective binding of A1 to a primer terminus on SSB-coated template DNA. The primed template-A1 complex, in the presence of ATP, then interacts with PCNA, forming a PCNA adduct that is stable to gel filtration. This DNA-A1-PCNA complex can then interact with pol δ , or pol ϵ , which, in the presence of dNTPs, catalyzes processive DNA synthesis. The interaction of Cip1 with PCNA could interfere with the binding of PCNA to A1, to pol δ , or to both. Cip1 complexed to PCNA could also impair the movement of the replication complex along the DNA. The data presented above suggest that probably each of these reactions can be affected by Cip1. Results supporting this suggestion include the following. (i) The most pronounced effect of relatively low levels of Cip1 (preincubated with PCNA prior to the addition of A1 and pol δ) was on the synthesis of long DNA products. (ii) Inhibition of the elongation of chains over a short 10-nt stretch required high molar ratios of Cip1 to PCNA (>40-fold). (iii) When PCNA was first loaded on to primed DNA by A1, subsequent incubation with Cip1 only slightly inhibited the 10-nt elongation reaction. This indicates that Cip1 can block the clamp-loading step when present at high molar ratios to PCNA. (iv) Preliminary experiments indicated that the loading of PCNA on A1-primed DNA was the same in the presence and absence of 2-fold molar excess of Cip1 (data not presented). These observations suggest that Cip1, when present at molar ratios to PCNA of 4:1 or lower, interferes with the rate of pol δ -catalyzed DNA synthesis. It could do this by interfering with only the sliding of the complex along DNA or by decreasing the association of pol δ with PCNA. The latter possibility, however, seems less likely since the inhibition by Cip1 was unaffected by supplementation of reactions with more pol δ , and preincubation of A1 and PCNA with the singly primed substrate prevented inhibition by Cip1 of the short-stretch elongation reaction.

The finding that Cip1 can alter rates of DNA synthesis may have significant implications for understanding cell cycle checkpoints. Previously checkpoints were thought to block major cell cycle transitions in order to prevent replication and segregation of damaged templates. Available data are consistent with a direct role for Cip1 in this p53-dependent G₁ checkpoint induced by DNA damage via its ability to associate with and inactivate cyclin E-Cdk2 complexes, which function at the G₁/S transition (7). The data presented here indicate that Cip1 may provide a second checkpoint function by directly inhibiting PCNA-dependent chain elongation catalyzed by pol δ . While PCNA has been shown to participate in repair reactions (23), it is likely that only very high levels of Cip1 complexed to PCNA would inhibit repair, because it involves the synthesis of relatively short lengths of DNA. The polymerase that participates in the repair of damaged DNA has been shown in some instances to be pol ϵ (24, 25), whose activity can utilize the PCNA sliding clamp (10, 26). We suspect that, as for pol δ , the elongation of primed templates by pol ϵ will also prove to be sensitive to Cip1. D-type cyclins have been shown to physically associate with PCNA in the absence of a Cdk (27). Thus, it is possible that

the affinity of Cip1 for PCNA could be substantially enhanced in the presence of Cdk-cyclin D complexes through the formation of multiple protein-protein interactions.

p53 has also been implicated in DNA damage-dependent apoptosis, and Cip1 is induced during this process (7). The decision to remain arrested or undergo apoptosis may depend in part on the extent of damage or the presence of conflicting growth control signals. Cip1, through its association with PCNA, could play a role in transducing signals leading to apoptosis, perhaps by permanently inhibiting DNA synthesis.

Several DNA tumor viruses express proteins that bind and inactivate p53. If replication of these agents were sensed as DNA damage by the cell, Cip1 could prevent their replication by sequestering PCNA. Thus the necessity to remove p53 may not only facilitate exit from G₁ to S but could also optimize cells for high-level viral DNA synthesis by blocking Cip1 formation. Whether Cip1 is involved in checkpoints, apoptosis, or both, it is likely that the alteration of the rate and extent of DNA synthesis by Cip1 is an important aspect of the cell's ability to regulate S phase in response to signals elicited by DNA damage.

Note Added in Proof. Recently, a cyclin-dependent kinase inhibitor implicated in G₁ phase arrest, p27^{Kip1}, has been isolated (28). This protein possesses some regions of sequence similarity to Cip1. However, recombinant p27^{Kip1} did not inhibit the elongation of primed DNA templates in reaction mixtures as described in Fig. 3, nor did it interact with PCNA, measured by SPR as described in Fig. 5A (K. Polyak, J. Massagué, A. Koff, Z.K., M.O., and J.H., unpublished results).

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A question of balance: the role of cyclin-kinase inhibitors in development and tumorigenesis

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and J. Wade Harper

Cyclin-kinase inhibitors (CKIs) are versatile negative regulators of cell proliferation that function in developmental decisions, checkpoint control and tumour suppression. Phenotypic examination of mice lacking individual CKIs has begun to reveal the specialized roles that each of these proteins play in vivo. This review focuses on what has been learned about the role of CKIs in development and cancer through the generation of knockout animals. The authors discuss whether differences in knockout phenotypes between CKIs reflect differential use of these inhibitors by the organism or a fundamental difference between the inhibitors, and suggest a balance hypothesis to explain the different effects observed.

similarity, and they differ in specificity and mechanism of inhibition^{4–6}. All of these inhibitors function to block the activity of G1 CDKs, but, unlike the CIP-KIP family, which effectively inhibits multiple classes of G1 kinases including CDK2, CDK3, CDK4 and CDK6, the INK4 family is specific for CDK4 and CDK6 (Fig. 1). When the first CKI was discovered in mammals, it was hypothesized that these molecules would play roles in development and differentiation, cell-cycle checkpoints and tumour suppression. These predictions have now been realized through the analysis of CKI-deficient mice. This review will focus on what has been learned about the role of CKIs in development and tumorigenesis. Owing to space limitations, a number of areas related to CKI biochemistry and function in tissue-culture cells have not been reviewed extensively here. Many of these topics are covered in previous reviews^{4–6}.

Linking CKIs to developmental events and cancer predisposition

An emerging theme in cancer research and developmental biology is that tissues that show a special dependency on particular genes to control their proliferation during development seem to be driven to malignancy by alterations in those same genes⁷. The finding that individual CKIs including p21^{Cip1/Waf1} and p57^{Kip2} are expressed in particular cell types during development suggests that these proteins contribute to maintenance of the nonproliferative state in a cell-type-specific manner^{8–10}. In general, these two CKIs are expressed at the highest levels in cells that are postmitotic and terminally differentiated (Table 1), although, in principle, these and other inhibitors may also function in the many starts and stops in proliferation that cells must undergo during a developmental programme.

The striking tissue- and cell-type-specific expression patterns observed for CKIs suggests that a variety of antimitogenic signalling pathways will influence the expression of particular target CKIs. Although induction of CKI expression is observed in a number of different *in vitro* differentiation systems (reviewed in Refs 4 and 5), it is not clear whether CKIs drive differentiation or whether their induction is a consequence of it. The two different possibilities have been observed *in vitro* and may be interlinked (see Fig. 2). Expression of p21 is induced by the vitamin D receptor in myelomonocytic cells, suggesting that certain CKIs can be direct targets of differentiation-inducing agents¹¹. In muscle cells, the transcription factor MyoD induces the expression both of the gene encoding p21 and of genes required for myotube formation^{8,12}. Expression of the bZIP protein C/EBP α in fibrosarcoma cells leads to increased amounts of p21, both through activation of the *Cip1* promoter and increased stability of p21 (Ref. 13). In addition, transforming growth factor β (TGF- β)-dependent activation of CKI expression, including p15^{Ink4b} and p21^{Cip1}, has also been observed (reviewed in Ref. 4). Evidence that CKIs can promote differentiation comes from the finding that ectopic expression of p21 or p27 in myelomonocytic cells leads to both cell-cycle arrest and induction of cell-surface

The development and maintenance of multicellular organisms requires precise temporal and spatial control of cell division. During development, mitogenic signals function within particular proliferative domains and are opposed by antimitogenic signals that bring about the cessation of proliferation. These signals are integrated through the activity of cyclin-dependent kinases (CDKs), enzymes composed of a CDK (the catalytic subunit) and an essential activator subunit called cyclin. CDKs are regulated by many mechanisms, reflecting both the diversity of signals they integrate and the central importance of their role in cell-cycle control. These regulatory mechanisms include variations in cyclin abundance¹, positive- and negative-acting phosphorylation of the kinase subunit^{2,3}, and the actions of cyclin-kinase inhibitory proteins [CKIs, also known as CDK inhibitory proteins (CDIs)]. Of these, the CKIs appear to be the most diverse and flexible regulators.

Two classes of CKIs – the INK4 family and the CIP-KIP family – have been defined based on sequence

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macrophage-specific markers indicative of differentiation¹¹. Thus, the relationship between CKI expression and differentiation may be one of interdependency and mutual reinforcement.

While tissue-culture systems are useful to analyse certain responses, cells in animals exist in a much more complex environment that is not recreated *in vitro*. Thus, the true significance of a CKI to the control of proliferation and development is perhaps best realized through analysis of CKI-deficient animals. In principle, this approach identifies cell types where a particular inhibitor makes a crucial contribution to the balance of negative and positive growth-control factors that must be maintained for homeostasis or proper development and identifies cell types where loss of a particular CKI leads to cancer predisposition. Furthermore, knockouts of multiple CKIs will ultimately reveal the cell types that possess redundant or alternative pathways for negative growth control.

To date, mice deficient in genes encoding p21^{Cip1}, p27^{Kip1} and p16^{Ink4a} have been reported, and together the phenotypes observed span the gamut of what one might expect for loss of a cell-cycle inhibitor. All three mice are viable, and display intermediate-to-severe phenotypes with respect to development and tumorigenesis (Table 2).

Cip/Kip-knockout mice – checkpoint deficiency and proliferative miscues

p21^{Cip1} is unique among the known CKIs in that it is utilized in the DNA damage response pathway and is a transcriptional target of the tumour-suppressor protein p53 (reviewed in Refs 4–6). Loss of p53 function leads to defects in the G1 checkpoint in response to DNA damage, the G2 checkpoint in response to spindle malfunction, and the apoptotic response of some cell types to DNA damage¹⁴. Some combination of these functions is thought to be necessary for tumour suppression by p53. Because of the central position occupied by p21 with respect to DNA damage and cell-cycle control, it seemed possible that p21 was required for some or perhaps all of the effects of p53. The generation of p21-knockout mice¹⁵ and p21-null chimeric mice¹⁶ provided an *in vivo* setting in which to test the contribution of p21 to p53 function. Unlike p53-deficient mice, which incur tumours with high frequency, p21-null mice do not develop spontaneous tumours at a frequency higher than that of wild-type mice¹⁵. This is consistent with the low frequency of p21 mutations in human cancers^{17,18}. Also, unlike p53-mutant cells, p21-deficient cells do not display alterations in apoptosis induced by DNA damage (thymocytes or epithelial cells)^{15,16} or in the G2 spindle checkpoint (fibroblasts)¹⁵. However, mouse fibroblasts lacking p21 are partially defective in the DNA-damage G1 checkpoint^{15,16}, a result confirmed in human cells¹⁹. These results indicate that other p53-dependent functions (e.g. apoptosis), alone or in combination with its checkpoint function, are the essential components of its tumour-suppressor activity. Additional studies are required to determine whether loss of p21 contributes to increased tumorigenesis induced by DNA damage.

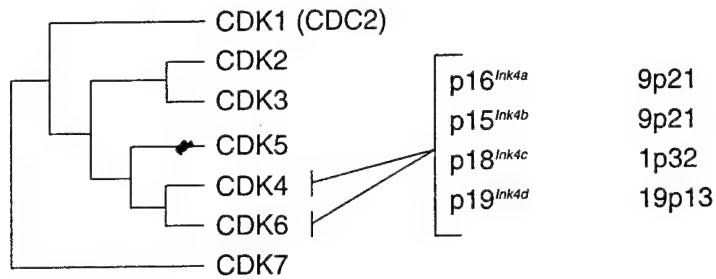
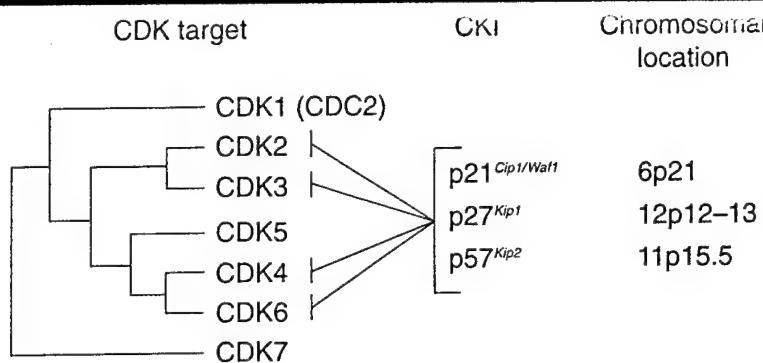


FIGURE 1

Kinase inhibitory specificity of the CIP/KIP and INK4 classes of cyclin-dependent kinase (CDK) inhibitors. A family tree of the known CDKs, as determined by sequence comparisons, is depicted along with the inhibitory specificity of each cyclin-kinase inhibitor (CKI). The chromosomal location of each CKI is also indicated.

In contrast to p21^{Cip1}, p27^{Kip1}-deficient mice display several obvious developmental phenotypes (Table 2)^{20–22}. First, p27-null mice are larger than wild-type or heterozygous litter mates, indicating a proliferative advantage in the absence of p27 function. Although most of the major organs are all larger in p27-null mice, the sizes of two organs that normally express the highest levels of p27 (thymus and spleen) were increased to the largest extent. Increased

TABLE 1 – MAJOR SITES OF EXPRESSION OF p21 AND p57 IN THE MOUSE BY *IN SITU* HYBRIDIZATION*

	p21	p57
Embryo	Muscle/dermomyotome D8.5–10	Muscle/dermomyotome D8.5–10
	Apical ectodermal ridge D10	Brain D10
	Respiratory epithelium D12.5–15.5	Migrating neuroblasts D10
	Skin D15.5	Rathke's pouch D10
	Hair follicles D15.5	Lens D13.5
Adult	Intestinal epithelium	Kidney (cortex)
	Stomach epithelium	Muscularis mucosae (stomach)
	Skeletal muscle	Skeletal muscle
	Liver	
	Prostate epithelium	

*The days post coitus (D) where expression has been observed are indicated. p21 expression is obtained from data in Ref. 8, and p57 from Ref. 10. Expression in the prostate was documented in Ref. 9.

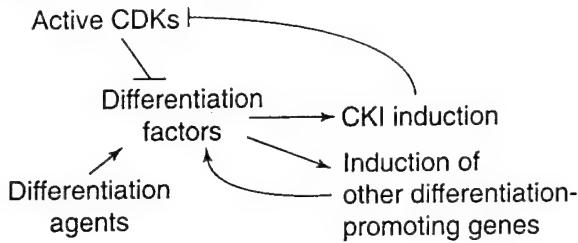


FIGURE 2

A model depicting a differentiation switch linking cyclin-dependent kinases (CDKs), differentiation factors and cyclin-kinase inhibitors (CKIs). The model is based on an analysis of activation of muscle-cell differentiation set in motion by MyoD, a transcription factor that is sufficient to specify the muscle-cell differentiation pathway^{8,13}. Activation of transcriptional targets of MyoD and vitamin D receptor leads to expression of certain CKIs (*Cip1* in these cases) as well as expression of additional genes required for establishment of the differentiated state. CKIs function presumably to inhibit CDKs, which may, directly or indirectly, maintain differentiation factors in an inactive state. Other genes may potentiate the activity of differentiation factors, which maintains the cell-cycle switch in the off configuration.

thymus size is correlated with increased proliferation in both the cortical and medullary regions, but neither the distribution of T-cell markers nor the extent of apoptosis is altered. Second, female p27-deficient mice are infertile, probably because of a lack of luteal cell differentiation, although a role for pituitary malfunction cannot be ruled out. Third, p27-null mice display defects in neural retina development²⁰. Since p27 is expressed selectively in the photoreceptor cells in the eye, it seems likely that the disorganized retinal phenotype results from loss of growth control. Finally, p27-null mice display intermediate lobe pituitary hyperplasia or adenoma, consistent with the increased size of this organ, but malignant pituitary tumours are rare. The low frequency of tumours in

p27-deficient mice is also consistent with the absence of mutations in the gene encoding p27^{Kip1} in human cancers^{23,24}.

A variety of tissue-culture experiments have implicated p27 as a central regulator of cellular responses to a large number of antimitogenic signals, including arrest induced by TGF- β (Ref. 25), rapamycin²⁶, growth factor deprivation^{27,28} and contact inhibition²⁵. However, analysis of cells from p27-null animals reveals that these pathways do not absolutely require p27 (Ref. 20). T lymphocytes from p27-null mice are responsive to cell-cycle arrest by both TGF- β and rapamycin²⁰, indicating that p27 is not required for these processes. Previous data indicate that total amounts of p27, or p27 in association with CDK2, increase in fibroblasts upon serum deprivation or contact inhibition^{25,27,28}. However, fibroblasts from p27-null mice do not display alterations in saturation density and are readily arrested upon serum deprivation²⁰. These differences bring into question the predictive value of *in vitro* experiments for determining the *in vivo* roles of inhibitors and emphasize the importance of mutational analysis of CKIs for determining non-redundant functions.

Mice deficient in p57^{Kip2} have not yet been reported. The human *KIP2* gene is located at 11p15.5 (Ref. 10), a region of the genome linked to Wilms tumour (*WT2*) and Beckwith-Wiedemann syndrome, which display embryonic tumours, organ overgrowth and sporadic cancers. Both of these diseases show evidence of the involvement of imprinting, where loss of heterozygosity or chromosomal translocations involve only the maternal allele. Interestingly, *KIP2* has recently been shown also to be imprinted in both humans^{29,30} and mice³¹, and is expressed preferentially from the maternal allele in all tissues tested except brain. Whether the *Kip2*-deficient mice will shed any light on these two diseases and the role of p57 in development and cancer remains to be seen.

TABLE 2 – PHENOTYPES OF CKI-DEFICIENT MICE*

Genotype	Developmental	Transformation	Cellular
<i>p21</i> ^{-/-}	None	None	G1 checkpoint defect High saturation density
<i>p27</i> ^{-/-}	Increased animal size and organ overgrowth with particular effects on spleen and thymus Female infertility Defect in luteal cell differentiation Disorganization of retina	Intermediate pituitary hyperplasia (adenoma)	No defect in response to TGF- β , rapamycin or contact inhibition
<i>Ink4a</i> ^{-/-}	Mild proliferative expansion of the spleen	High incidence of fibrosarcoma and lymphoma Increased susceptibility to tumour induction by carcinogens	Increased sensitivity to transformation by Ha-Ras Failure to senesce High saturation density Decreased doubling time

*Abbreviation: TGF- β , transforming growth factor β .

***p16^{Ink4a}*, a component of a pathway frequently targeted for transformation**

The potential involvement of *p16^{Ink4a}* in the development of tumours was suggested initially by the finding that the gene encoding it maps to a chromosomal region frequently mutated in human cancers and by the fact that it was mutated or repressed by methylation in a high percentage of tumour-derived cell lines and a substantial fraction of primary tumour samples (Ref. 32; reviewed in Ref. 33). It is not surprising, therefore, that *Ink4a*-deficient mice develop spontaneous tumours at an early age and are highly sensitive to carcinogens³⁴. However, examining the unique involvement of *p16^{Ink4a}* in tumour suppression at this locus was

complicated by the fact that the gene encoding the related CKI p15^{Ink4b} is located adjacent to the *Ink4a* locus, and this gene is also frequently deleted along with *Ink4a* in primary tumours and in cell lines in which *Ink4a* is deleted. Furthermore, the *Ink4a* locus can produce two transcripts derived from two distinct promoters³⁵. The alternative transcript produces a protein called p19^{Arf} (for: alternative reading frame). mRNAs for p16^{Ink4a} and p19^{Arf} contain different 5' exons (E1 α and E1 β , respectively) but share common second and third exons that are translated in different reading frames. Although the mechanism does not involve CDK association, p19^{Arf} blocks cell-cycle progression in both G1 and G2 phases when over-expressed³⁵. In addition, Arf is expressed more ubiquitously than is *Ink4a* as judged by probe-specific northern analysis.

While a role for p19^{Arf} is not ruled out by the mutational data collected on the *Ink4a* locus, much of the data is consistent with the primary involvement of p16, particularly in familial melanoma, pancreatic cancer and oesophageal cancer. Approximately 50% of all mutations affect p16 alone, with the remaining fraction affecting both p16^{Ink4a} and p19^{Arf}; mutations found in familial melanoma kindreds are most frequently in E1 α (reviewed in Ref. 33). Many nonsense mutations specifically affect p16, and a number of missense mutations in p16 have been shown to affect association with CDK4/CDK6 and cell-cycle arrest^{36,37}, some in a temperature-sensitive fashion³⁸. However, the fact that there are four known missense mutations in E2 that affect p19^{Arf} but not p16^{Ink4a} leaves open the possibility that p19^{Arf} contributes to tumour suppression in some cell types³⁵, although these alleles have not been shown to be defective in growth suppression.

Deletion of exons 2 and 3 has no effect on mouse embryo viability³⁴, indicating that neither p16^{Ink4a} nor p19^{Arf} is required for development. However, young *Ink4a*-null mice display a mild proliferative expansion of the spleen, indicative of an abnormal extramedullary process. This suggests a role for p16^{Ink4a} in control of proliferation and/or differentiation in particular haematopoietic lineages. The absence of a strong developmental phenotype might have been predicted since two cases of p16-null humans have been reported that do not have obvious developmental defects³⁹.

While it is no surprise that *Ink4a*-null mice are susceptible to spontaneous and carcinogen-induced cancer, the tissue spectrum observed is somewhat surprising. Although the number of spontaneous tumours analysed is still relatively small, fibrosarcomas and lymphomas are by far the most common³⁴. In humans, both of these tumour types have frequent p16 mutations³³. However, melanoma, pancreatic or oesophageal tumours, which are frequent in humans, have not been detected yet in *Ink4a*-deficient mice³⁴. This reiterates the difference in tumour-type specificity that is frequently observed when mice are compared with humans. For example, mice lacking the retinoblastoma protein Rb develop pituitary tumours, but not retinoblastoma as found in humans (reviewed in Ref. 40). The strength of the

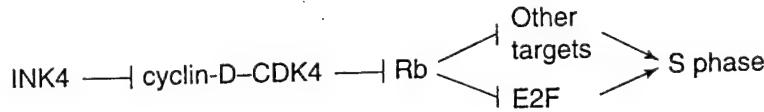


FIGURE 3

The cyclin-D-Rb pathway is targeted for mutation in a large percentage of human tumours. Rb functions to block the action of a number of proteins, such as E2F, required for S-phase entry. Phosphorylation of Rb by cyclin-D-CDK4, and perhaps other cyclin-dependent kinases (CDKs), is required for Rb inactivation. INK4 homologues function to block the action of cyclin-D-CDK4, thereby impeding entry into S-phase. Loss-of-function mutations in either Rb or INK4 occur in a large number of tumours. In addition, cyclin D1 is overexpressed in some tumour types, thereby decreasing the effectiveness of p16^{INK4}. In addition, mutations in CDK4 that block inhibition by INK4 homologues have also been observed⁴⁴, thereby providing an alternative mechanism for bypassing p16^{INK4} function.

tumour-suppressor function of *Ink4a* is indicated by the low survival rate, which is comparable to that observed for p53-null mice (reviewed in Ref. 41).

INK4 family members participate in a genetic pathway that includes cyclin-D-CDK4, Rb and E2F (Fig. 3)^{4,6,42}. Rb acts to restrain the action of the E2F family of transcription factors, which coordinate the transcriptional programme of genes involved in DNA synthesis, and association with E2F may also allow Rb to function as a repressor of growth-promoting genes (reviewed in Ref. 43). Rb appears to be the sole essential substrate of CDK4-cyclin-D complexes. Therefore, mechanisms that lead to unregulated Rb kinase activity, including overexpression of cyclin D and disruption of the inhibitory function of p16^{Ink4a} [through mutation in either *Ink4* or CDK4 (Ref. 44)], facilitate inappropriate Rb inactivation and cell-cycle entry. Thus, this pathway serves as a single genetic target for transformation, and its disruption may be required for the genesis of the majority of human cancers.

The balance hypothesis

Mice defective in p16^{Ink4a} (or the Rb pathway in general) display high rates of tumorigenesis, whereas loss of the p21 or p27 inhibitors leads to, at most, hyperplasia. This brings into focus the question of the biochemical and physiological basis for this difference. Is this merely a reflection of how an organism differentially utilizes two classes of equivalent inhibitors? Does the p16 pathway happen to be the primary means used to maintain the differentiated and nonproliferative state, while p21 and p27 are used primarily to control proliferation during development? Or does the answer lie in the distinct biochemical properties characteristic of each class of inhibitors? We would like to explore this latter possibility and put forward a speculative model termed the 'balance hypothesis' to explain the phenotypic differences observed in these mutants. In this model, the constellation of active cyclin-CDK complexes resulting from the loss of particular inhibitors results in different consequences for genomic stability and tumorigenesis.

While both classes of CKIs may contribute to growth control in a tissue-specific manner, their loss might result in significantly different effects that reflect the distinct inhibitory specificities of each class.

CIP/KIPs inhibit both CDK4/CDK6 and CDK2/CDK3 kinases in a cyclin-dependent fashion. Loss of these inhibitors might simultaneously elevate the kinase activity of both cyclin-D- and cyclin-E-dependent kinases, producing a state that mimics a normal S-phase entry. By contrast, p16 is known to inhibit specifically only the cyclin-D-dependent kinases, CDK4 and CDK6 (Fig. 1), and its loss would activate predominantly these cyclin-D-dependent kinases, leading to Rb inactivation and release of bound factors that promote S-phase entry (e.g. E2F). It is known that overproduction of E2F can cause S-phase entry without full cyclin-E-CDK2 activation⁴⁵. However, this S phase is defective and results in apoptosis. The balance hypothesis predicts that for an optimal, error-free S phase, the cell requires coordinate activation of both cyclin E and cyclin D kinases (i.e. coordinated activation of cyclin-E-CDK2 and inactivation of Rb). Mutants in the p16-cyclin-D-Rb pathway would produce an imbalanced activation of S phase, resulting in genomic instability and increased rates of aneuploidy, chromosomal rearrangements or point mutations. Consistent with this notion, diploid fibroblasts expressing the E7 protein of human papillomavirus, which binds and inactivates Rb, display hallmarks of genomic instability including aneuploidy as a mechanism of drug resistance^{46,47} and enhanced rates of karyotypic abnormalities⁴⁸. Thus, loss of the Rb pathway results not only in reduced growth control, but also in an acceleration in the rate of accumulation of genetic alterations that further reduces proliferation restraints. This interpretation relies on the assumption that the main effects of E7 are mediated via inactivation of Rb and not additional pathways.

It is, perhaps, this difference in balance through which p16 loss may exert its profound oncogenic potential. While this model remains speculative, it does generate certain testable hypotheses. For example, p16- or Rb-deficient mouse embryonic fibroblasts should show signs of genomic instability. Furthermore, the relative instability should be enhanced by reducing cyclin-E-CDK2 activity (i.e. enhancing the imbalance). It is not clear whether the converse would also be true, increasing cyclin-E-CDK2 activity would reduce instability. This is because those conditions would not restore the relative timing of the inactivation of Rb and the activation of cyclin E kinases that occurs in a normal cell cycle and that, too, may be important to genomic stability. If the balance hypothesis is true, it may have implications for many oncogenic events. Since inappropriate activation of the cell cycle by a mutagenic event, such as proto-oncogene activation, is unlikely to precisely recreate a normal balance of kinases, it is possible that many of these events will also have effects on genomic stability, and this may be an important aspect of their oncogenic potential.

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Inhibition of Cyclin-dependent Kinases by p21

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p21^{Cip1} is a cyclin-dependent kinase (Cdk) inhibitor that is transcriptionally activated by p53 in response to DNA damage. We have explored the interaction of p21 with the currently known Cdks. p21 effectively inhibits Cdk2, Cdk3, Cdk4, and Cdk6 kinases (K_i 0.5–15 nM) but is much less effective toward Cdc2/cyclin B (K_i ~400 nM) and Cdk5/p35 (K_i >2 μ M), and does not associate with Cdk7/cyclin H. Overexpression of p21 arrests cells in G1. Thus, p21 is not a universal inhibitor of Cdks but displays selectivity for G1/S Cdk/cyclin complexes. Association of p21 with Cdks is greatly enhanced by cyclin binding. This property is shared by the structurally related inhibitor p27, suggesting a common biochemical mechanism for inhibition. With respect to Cdk2 and Cdk4 complexes, p27 shares the inhibitory potency of p21 but has slightly different kinase specificities. In normal diploid fibroblasts, the vast majority of active Cdk2 is associated with p21, but this active kinase can be fully inhibited by addition of exogenous p21. Reconstruction experiments using purified components indicate that multiple molecules of p21 can associate with Cdk/cyclin complexes and inactive complexes contain more than one molecule of p21. Together, these data suggest a model whereby p21 functions as an inhibitory buffer whose levels determine the threshold kinase activity required for cell cycle progression.

INTRODUCTION

Passage through the eukaryotic cell cycle is regulated by the progressive activation and inactivation of a family of cyclin-dependent protein kinases (Cdks)¹ (reviewed in Draetta, 1993; Pines, 1993; Hunter and Pines, 1994; Sherr, 1994). The temporal activation of individual Cdks is dictated in part by the timing of expression of their cognate cyclins together with both

activating and inhibitory phosphorylation (reviewed in Sherr, 1994). The best characterized cyclins fall into four classes (D, E, A, and B type) and are required in the G1, G1/S, S, and G2/M phases of the cell cycle, respectively (reviewed in Sherr, 1994). A number of Cdks, including Cdc2, Cdk2, Cdk4, and Cdk6 have been characterized with respect to their temporal activation and cyclin partners (Matsushime *et al.*, 1992; Meyerson and Harlow, 1994; reviewed in Pines, 1993 and Sherr, 1994).

An additional layer of Cdk regulation has emerged with the discovery of the Cdk inhibitors p21, p27, p16, and p15 (reviewed in Elledge and Harper, 1994; Hunter and Pines, 1994; Nasmyth and Hunt, 1993; Peter and Herskowitz, 1994). p21^{Cip1} is a potent inhib-

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¹ Abbreviations used: Cdk, cyclin-dependent kinase; DTT, dithiothreitol; GST, glutathione S-transferase; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

itor of Cdk2 and Cdk4 kinase complexes in vitro (Gu *et al.*, 1993; Harper *et al.*, 1993; Xiong *et al.*, 1993b), is induced in senescent cells (Noda *et al.*, 1994), and is transcriptionally regulated by p53 (El-Deiry *et al.*, 1993). p21 is induced by DNA damage in a p53-dependent manner and is found to be associated with inactive cyclin E/Cdk2 complexes (Dulic *et al.*, 1994; El-Deiry *et al.*, 1994), consistent with a role in mediating the p53-dependent cell cycle arrest. In some fibroblast cell lines, including those from Li-Fraumeni cells homozygous for mutant p53 (Li *et al.*, 1994), p21 expression is largely dependent on p53. However, p21 expression in embryonic and adult mice is p53 independent (Parker *et al.*, 1995), indicating that the regulation of p21 is more complex than originally thought.

p21 is found in complexes with the replication factor PCNA in extracts from normal cells (Xiong *et al.*, 1992; Zhang *et al.*, 1993) and can associate directly with PCNA in vitro (Florez-Rozas *et al.*, 1994; Waga *et al.*, 1994). Association of p21 with PCNA leads to inhibition of PCNA-dependent DNA synthesis catalyzed by DNA polymerase δ and RF-C in vitro. The ability to inhibit DNA replication has been hypothesized to be part of a checkpoint during S phase that is activated by DNA damage to prevent replication beyond potentially mutagenic lesions while allowing repair processes time to eliminate damage.

p27^{Kip1} (Polyak *et al.*, 1994; Toyoshima and Hunter, 1994) is structurally related to p21 and inhibits both Cdk2 and Cdk4 in vitro. It is not regulated by p53 and does not associate with PCNA in vitro (Flores-Rozas *et al.*, 1994), suggesting specialization for a non-checkpoint function. In contrast, p16 and p15, which form a family of structurally related inhibitors distinct from the p21 family, are apparently specific for Cdk4 and Cdk6 (Serrano *et al.*, 1993; Hannon and Beach, 1994; Jen *et al.*, 1994).

Although p21 is found to be associated with several Cdk complexes in normal diploid fibroblasts, its role in proliferating cells is not well understood. For example, it is not known whether p21 associates tightly with monomeric Cdks, thus inhibiting a percentage of cyclin/Cdk complexes as they form, or whether p21 binds preferentially to cyclin bound kinases, thereby acting as a titratable buffer. Furthermore, the cellular targets remain unknown. Does p21 inhibit all Cdks, or only a subset and with what affinities? In this paper, we examine the interaction of all known Cdk family members with p21. The data indicate that p21 preferentially inhibits Cdks that function in the G1 and S phases, consistent with our finding that cells overexpressing p21 arrest with a G1 DNA content. In addition, we have observed that the affinity of both p21 and p27 for Cdks is greatly enhanced by cyclin association. Moreover, greater than 95% of the active Cdk2 in normal diploid fibroblasts contain bound p21 but can be completely inactivated by exogenous p21. The

data presented in this paper are consistent with the notion that p21 acts as a cell cycle buffer that must be titrated to exit G1.

MATERIALS AND METHODS

Expression Plasmids and Insect Cell Viruses

pET-p21 and GST-p21 were from a previous study (Harper *et al.*, 1993). pET-HAp21 was constructed by ligating the p21 coding sequence into pET-HA (Elledge *et al.*, 1992). This plasmid produces an N-terminal fusion of p21 containing a 16-amino acid hemagglutinin A epitope tag. pCMV-p21 was constructed by ligating a 1.6-kb *Xba*I/*Bgl*II fragment from p21 into pCMV. pCMV-CD20 and pCMV-Cdk2-DN (containing the mutation Asp-145 \rightarrow Asn) were provided by Dr. S. van der Heuvel. pCMV-Rb was provided by Dr. S. Dowdy. pET21a(+)p27 containing the mouse p27 open reading frame fused to a C-terminal six-histidine tag was provided by Dr. J. Massague (Sloan Kettering Institute, New York, NY). p21 deletions were generated by polymerase chain reaction (PCR) using a single 5' primer (containing an *Nde*I site at the initiation codon) and a nested set of 3' primers (containing a *Bam*HI site and a stop codon) to give truncations at amino acids 60, 80, 100, 120, and 140. PCR fragments were ligated into pCRII and appropriate fragments were subcloned into a modified version of pGEX2TK. pCRII-p27 for in vitro translation was obtained by ligating the p27 open reading frame (obtained by PCR using a mouse cDNA library and primers flanking the start and stop codons) into pCRII (Invitrogen, San Diego, CA). For expression of p21, GST-Cdk4, GST-cyclin E, and His₆-tagged Cdk3 in sf9 cells, appropriate open reading frames were subcloned into either pVL1393 or pBlueBacHis. Viruses were generated using Baculogold (PharMingen, San Diego, CA) as recommended by the supplier. Other viruses (Cdk2HA, Cdc2, Cdk2, Cdk4, Cdk6, cyclin A, GST-cyclins A and B, and cyclins D1, D2, and D3, Cdk7HA, and cyclin H) were generously provided by either Drs. H. Piwnica-Worms (Washington University, St. Louis, MO), C. Sherr (St. Jude's Children's Research Hospital, Memphis, TN), M. Meyerson (Massachusetts General Hospital Cancer Center, Charlestown, MA), and D. Morgan (University of California, San Francisco, CA). Cyclin A and HA-Cdk2 purified to homogeneity from *Escherichia coli* are from a previous study (Connell-Crowley *et al.*, 1993).

Antibodies

Polyclonal antibodies against p21 or p27 were generated either in rabbits or mice using proteins purified from *E. coli*. Anti-p21 antibodies were affinity purified using GST-p21 covalently cross-linked to glutathione-Sepharose. Antibodies against the C-terminus of p21 (anti-p21^C) were generated using the peptide CKRRLIFSKRKP and affinity purified using immobilized GST-p21. Anti-cyclin A (Elledge *et al.*, 1992), anti-Cdk5 (Tsai *et al.*, 1993), and anti-p35 (Tsai *et al.*, 1994) were from previous studies. Other antibodies were purchased from Santa Cruz (Santa Cruz, CA; anti-Cdk4, anti-Cdk2), PharMingen (anti-CyD1 and anti-CyD2), Babco (Richmond, CA; anti-HA), or Becton-Dickinson (San Jose, CA; anti-CD20).

Protein Expression and Purification

p21, HA-p21, and p27-His6 (Polyak *et al.*, 1994) were expressed in strain BL21(DE3) as described (Harper *et al.*, 1993) except that induction was performed at 25°C. For purification, cells from 250 ml of culture were suspended in 17 ml of lysis buffer (LS: 25 mM Tris-HCl [pH 8], 25 mM NaCl, 10% sucrose, 5 μ g/ml leupeptin, 5 μ g/ml antipain, 1 mM phenylmethylsulfonyl fluoride) containing 0.1 mg/ml lysozyme and the cells were lysed by sonication. Cell suspensions were boiled for 10 min and insoluble proteins were removed by centrifugation. Approximately 10% of p21 present in total *E. coli* lysate was soluble and >80% of this p21 remained soluble after boiling. Extracts were passed over a HighTrap-Q col-

umn (Pharmacia, Piscataway, NJ) and the dropthrough applied to a HighTrap-S column. After washing with column buffer (CB: 25 mM Tris-HCl [pH 8], 2 mM EDTA, 5 mM DTT) containing 0.2 M NaCl, p21 and HA-p21 proteins were eluted with CB containing 0.4 M NaCl (p27 was eluted with 0.2 mM NaCl). Protein purity of such preparations was 90–95% for p21 and ~98% for p27. Removal of trace contaminating proteins from p21 preparations was achieved by chromatography on a MonoS column using a linear gradient of NaCl in 25 mM Tris-HCl (pH 8), 2 mM EDTA, 5 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 1 μ g/ml leupeptin (25–600 mM NaCl in 60 min). p21 eluted at ~450 mM NaCl and is greater than 98% pure by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were stored at -70°C in elution buffer containing 10% glycerol. Protein concentrations were estimated by Bradford assays (Bio-Rad, Richmond, CA) using bovine serum albumin as a standard. GST-Rb, GST-p21, and GST-p21 deletion proteins were expressed and purified as described previously (Harper *et al.*, 1993). Purified Erk2 was provided by Dr. M. Cobb (University of Texas Southwestern, Dallas, TX). A 56-kDa C-terminal fragment of Rb purified from *E. coli* was provided by Dr. D. Goodrich (M.D. Anderson Cancer Center, Houston, TX).

GST-cyclin A/Cdk2, GST-cyclin E/Cdk2, and GST-cyclin B/Cdc2 were purified after co-infection of sf9 cells as described (Harper *et al.*, 1993). GST-Cdk4/cyclin D2 was purified after co-infection using buffers containing Tween-20 as described (Matsushime *et al.*, 1994). Proteins were stored in glutathione elution buffer containing 10% glycerol at -70°C. For some experiments, GST-cyclin A/Cdk2 metabolically labeled with [³⁵S]methionine (see below) was removed from the GST protein by cleavage with thrombin. Gel filtration of purified Cdk2/cyclin A complexes was performed on a Pharmacia Superose-12 column as described previously (Gu *et al.*, 1993) at a flow rate of 0.3 ml/min.

Crude insect cell lysates containing activated Cdk/cyclin complexes, individual components, or p21 were prepared by lysing ~2 \times 10⁶ cells in 200 μ l of 50 mM HEPES (pH 7.5), 10 mM MgCl₂, 10 mM DTT, 0.5 mM NaF, 0.1 mM sodium vanadate, 5 μ g/ml leupeptin, 5 μ g/ml antipain (Matsushime *et al.*, 1992) and the solution was brought to 150 mM NaCl before centrifugation (14,000 \times g for 10 min). Extracts were stored at -70°C. Proteins were labeled with [³⁵S]methionine at 40 h post-infection using established procedures (Matsushime *et al.*, 1992).

Kinase Assays

Routine histone H1 kinase assays were performed in 65 mM potassium- β -glycerophosphate (pH 7.3), 15 mM MgCl₂, 16 mM ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 10 mM DTT containing 1 mg/ml ovalbumin with 2.5 μ M histone H1, 40 μ M [γ -³²P]ATP (0.2–0.8 nCi/pmol), and the indicated amounts of kinase and inhibitor proteins (37°C, 15 min-1 h). For GST-Cdk4 assays, a 56-kDa C-terminal Rb fragment was used as substrate. Reactions were stopped with 2X SDS sample buffer, electrophoresed in 12% SDS-polyacrylamide gels, and the dried gels were subjected to autoradiography. Unless otherwise noted, gel quantitation was achieved using a Molecular Dynamics Phosphoimager (Sunnyvale, CA). In some cases, histone H1 peptide at 50 μ M was substituted for histone H1 and assayed using filter binding as described (Harper *et al.*, 1993). GST-Rb kinase assays for Cdk3 and Cdk6 were performed using crude insect cell lysates containing activated cyclin/Cdk complexes or individual components as described previously (Matsushime *et al.*, 1992; Harper *et al.*, 1993). For experiments involving Cdk5, C33A cells were cotransfected with pCMV-Cdk5 and pCMV-p35 or with pCMV using calcium phosphate and cell lysates were subjected to immunoprecipitation using normal rabbit serum, anti-Cdk5, anti-p35, or anti-Cdk2 (Tsai *et al.*, 1993, 1994). Immune complexes were used for histone H1 kinase assays as described previously (Tsai *et al.*, 1993).

In Vitro Binding

For in vitro binding experiments employing crude sf9 cell extracts, 10 μ l aliquots of the indicated [³⁵S]methionine-labeled sf9 lysates or in vitro translation products were mixed on ice for 10 min. The total concentration of [³⁵S]methionine-labeled insect cell proteins was maintained by addition of extracts from sf9 cells infected with a nonrecombinant baculovirus. Binding buffer (150 μ l, 50 mM Tris-HCl [pH 8.0] containing 150 mM NaCl, 2 mM EDTA, 1 mM DTT, 0.5% NP40, 10 mM NaF, 0.1 mM sodium vanadate, 5 μ g/ml leupeptin, 5 μ g/ml antipain), 10 μ l of protein A-Sepharose, and 5 μ l of the appropriate antibody was added and the mixture was incubated at 4°C while rotating (60 min). Immune complexes were washed three times with 1 ml of binding buffer and bound proteins were analyzed by 13% SDS-polyacrylamide gels and autoradiography.

For binding experiments using purified kinases, [³⁵S]methionine-labeled cyclin A/Cdk2 was purified from sf9 cell co-infections as described above. A constant amount of cyclin A/Cdk2 (~50 ng Cdk2) was mixed with varying quantities of HA-p21 protein purified from *E. coli* in a total of 30 μ l of 65 mM potassium- β -glycerophosphate (pH 7.3), 15 mM MgCl₂, 16 mM EGTA, 10 mM DTT containing 1 mg/ml ovalbumin. After 20 min at 4°C, 150 μ l of binding buffer was added and proteins were immunoprecipitated with the indicated antibodies as described above and analyzed by SDS-PAGE. For kinase assays, 20% of each immune complex was washed with 1 ml of 20 mM Tris-HCl (pH 8.0) containing 15 mM MgCl₂ and histone H1 peptide kinase reactions were performed as described above. To examine whether p21 or p27 can associate directly with cyclin A, *E. coli* cyclin A (80 nM) was immunoprecipitated with anti-cyclin A antibodies in the presence or absence of 300 nM p21 or p27 in a total vol of 150 μ l of binding buffer. Immune complexes were subjected to immunoblotting using anti-p21 or anti-p27 antibodies and ECL detection.

Cell Culture, Transfections, and In Vivo Association

Human diploid fibroblasts (WI38), SV40 transformed human fibroblasts (VA13), and SAOS-2 osteosarcoma cells were grown in Dulbecco's minimal essential medium containing 10% fetal calf serum at 37°C. Calcium phosphate-mediated transient transfections (16 h) were performed using a total of 30 μ g of DNA containing 2 μ g of pCMV-CD20 and 28 μ g of either pCMV, pCMV-Cdk2(DN), pCMV-p21, or pCMV-Rb. Sixty-four hours post-transfection, cells were processed for flow cytometry as described (van der Heuvel and Harlow, 1993; Zhu *et al.*, 1993). Briefly, cells were washed with phosphate-buffered saline and stained with fluorescein isothiocyanate conjugated anti-CD20 antibodies (Becton-Dickinson). Washed cells were then fixed with 80% ethanol, and the DNA was stained with a propidium iodide solution (20 μ g/ml) containing 250 μ g/ml of ribonuclease A before flow cytometry using a Becton-Dickinson FACScan. DNA content in 4000–6000 CD20 positive cells is presented in the DNA histograms.

[³⁵S]Methionine labeling (100 μ Ci/ml; 6 h) was performed with the use of WI38 cells at 70% confluence using established procedures (Xiong *et al.*, 1992). To prepare cell lysates, cells were washed with phosphate-buffered saline and lysed in binding buffer containing 0.4% NP40, 50 mM NaF, 0.1 mM sodium vanadate, before centrifugation (10 min, 14,000 \times g, 4°C). Aliquots were subjected to sequential immunoprecipitation using normal mouse sera (6 μ l), anti-p21 sera (M831 or M832, 6 μ l), or anti-Cdk2 (Santa Cruz, 5 μ l) as described in the legend to Figure 6. Immune complexes were washed three times with 1 ml of binding buffer and once with 1 ml of kinase buffer. Twenty percent of each immune complex was taken for histone H1 kinase assays as described above and the remainder analyzed on 13% SDS-polyacrylamide gels. Dried gels were amplified and subjected to autoradiography.

RESULTS

p21 Preferentially Inhibits Cdk5 Involved in G1 and S Phases

To date, six members of the Cdc2 subfamily of protein kinases (Cdc2 and Cdk2, 3, 4, 6, and 7) have been shown to bind one or more proteins with homology to cyclins (reviewed in Sherr, 1994). Cdk5 is activated by a brain-specific protein p35, which displays no obvious sequence identity with known cyclins (Lew *et al.*, 1994; Tsai *et al.*, 1994). Previously, we and others demonstrated that p21 functions as an inhibitor of Cdc2, Cdk2, and Cdk4 kinase complexes in vitro and associates with Cdk2, Cdk4, and Cdc2 in extracts from normal diploid fibroblasts (Xiong *et al.*, 1992, 1993a,b; Gu *et al.*, 1993; Harper *et al.*, 1993). To characterize more fully the specificity of p21 for inhibition of Cdk family members, we have examined the interaction of p21 with all of the known Cdks.

The question of whether p21 is an inhibitor of Cdk3 and Cdk6 was addressed using kinase complexes assembled in sf9 cells. Cdk6 is activated by D-type cyclins (Meyerson and Harlow, 1994). Although the cyclin partner for Cdk3 has not yet been identified, its close sequence relationship with Cdk2 and its putative role in G1 (van der Heuvel and Harlow, 1993) suggested that it might be activated by cyclin E. As shown in Figure 1, co-expression of cyclin E and Cdk3 in sf9 cells results in assembly of active complexes. Both Cdk3 and Cdk6 are potently inhibited by purified p21 in Rb-kinase assays (Figure 1). To avoid inhibitor depletion, low levels of kinase (~ 1 nM) were used and under these conditions an apparent K_i value of ~ 15 nM for both Cdk3 and Cdk6 was determined. For comparison, the apparent K_i value for Cdk4/cyclin D1 in crude lysates was ~ 40 nM. These values represent upper limits for K_i because the interaction of insect cell proteins with p21 could potentially influence its availability or activity (see below). In contrast with these kinases, 500 nM p21 does not inhibit Cdk5/p35 complexes generated by cotransfection of p35 and Cdk5 expression plasmids in C33A cells (Figure 1C). As expected, Cdk2 kinase complexes from these cells were fully inhibited by 50 nM p21 (Figure 1C). In experiments using anti-Cdk5 immune complexes from mouse brain extracts, Cdk5 was not inhibited with 2 μ M p21 (our unpublished data). Experiments presented below show that p21 does not associate efficiently with Cdk7/cyclin H.

Previous studies have shown that p21 can inhibit Cdk2, Cdk4, and Cdc2 but the K_i values for these kinases have not been determined. Accurate K_i values cannot be reliably determined using crude extracts or immune complexes. To determine K_i values for the various Cdk/cyclin complexes, assays were performed using purified components under equilibrium conditions and inhibition plots were analyzed by non-

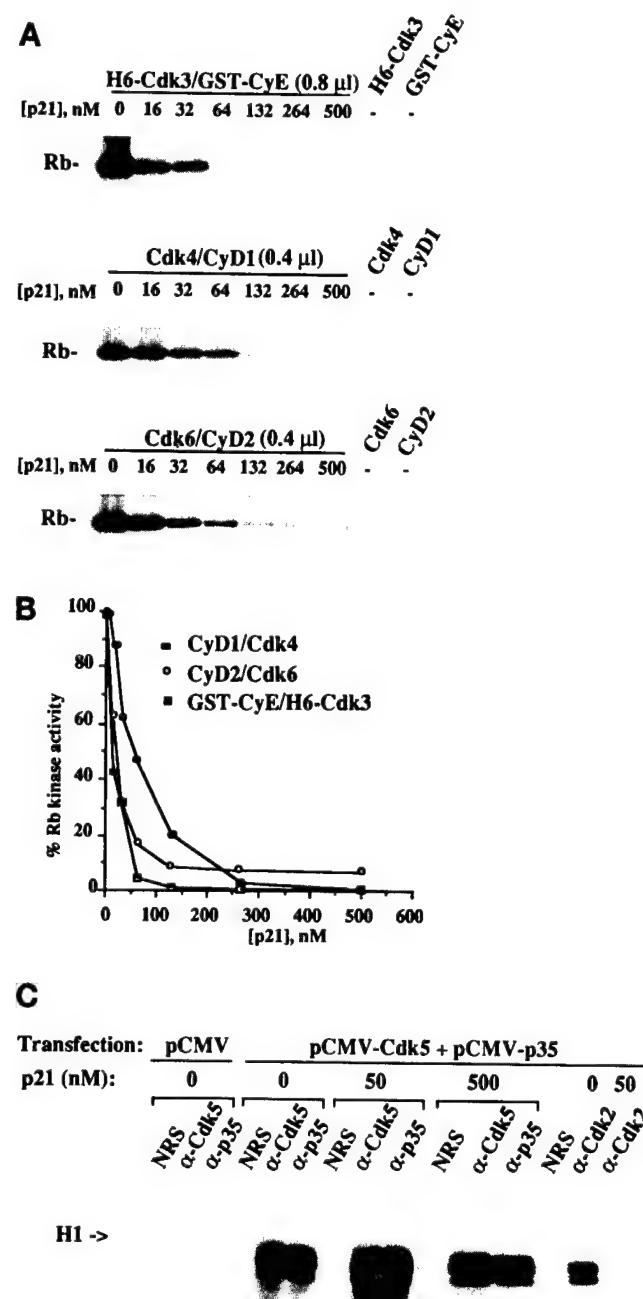


Figure 1. Inhibition of Cdk3, Cdk4, and Cdk6 by p21 in vitro. (A) GST-Rb kinase assays were performed using sf9 extracts from cells infected with the indicated baculoviruses and the indicated concentrations of p21 purified from *E. coli* (see MATERIALS AND METHODS). (B) Quantitation of Rb kinase assays was performed by liquid scintillation counting of bands excised from dried SDS gels. (C) Cdk5/p35 kinase activity is not inhibited by p21. Anti-Cdk5, anti-p35, or anti-Cdk2 immune complexes were prepared from C33A cells transfected with either pCMV or pCMV-Cdk5 and pCMV-p35 as described under MATERIALS AND METHODS and the immune complexes were split into equal portions before addition of the indicated amount of *E. coli* p21. Samples were then assayed for histone H1 kinase activity.

linear least squares. GST-cyclin E/Cdk2, GST-cyclin A/Cdk2, GST-cyclin B/Cdc2, and cyclin D2/GST-Cdk4 were purified from sf9 cells (Figure 2A) and used in assays with either histone H1 (Cdc2, Cdk2) or a C-terminal fragment of Rb (Cdk4) as soluble substrates. The concentrations of Cdk employed (as estimated by Coomassie Blue staining of SDS-gels using bovine serum albumin as a standard) was typically between 0.4–1 nM (Figure 2, legend). The apparent K_i value for inhibition of GST-cyclin A/Cdk2 by p21 is 0.5 nM. As expected based on previous studies (Harper *et al.*, 1993), cyclin B/Cdc2 is poorly inhibited by p21 (Figure 2B); the apparent K_i is ~400 nM and at 2 μ M, substantial activity (15%) remains.

The primary cellular targets of the p21 homologue, p27, are cyclin E/Cdk2 and cyclin D/Cdk4 complexes (Polyak *et al.*, 1994; Toyoshima and Hunter, 1994). To compare the potency of p21 and p27 toward these targets, assays were performed with p27 purified from *E. coli* using a procedure identical to that used for p21 (Figure 2A). The K_i value for inhibition of Cdk2/cyclin E by p27 (0.2–0.5 nM) was at least eightfold lower than that found for p21 (3.7 nM) (Figure 2C). In contrast, p21 is approximately fourfold more active toward cyclin D2/Cdk4 ($K_i = 0.6$ nM) than is p27 ($K_i = 4.7$ nM) (Figure 2D). The K_i value for Cdk4 determined using pure components is 40-fold lower than the apparent K_i determined using crude extracts (Figure 1 and Harper *et al.*, 1993), indicating that the use of crude extracts can lead to an overestimate of K_i values. In cases where K_i is near [E], significant inhibitor depletion can occur, in which case the true K_i value could be lower.

Together, these results indicate that p21 most effectively inhibits those Cdk family members involved in G1 and S-phase transitions. In addition, p21 and p27 show clear differences in affinity toward their primary cellular targets. Although it is possible that these proteins also inhibit other classes of kinases, high concentrations of p21 (1 μ M) do not inhibit protein kinase A (Harper *et al.*, 1993) or the MAP kinase Erk2 (our unpublished data).

The Amino Terminal 80 Residues of p21 Are Sufficient for Inhibition of Cdk2

C-terminal deletion analysis was used to identify a region of p21 capable of inhibiting cyclin A/Cdk2 in vitro. Coding sequences for the indicated p21 fragments were expressed as GST fusions and the purified proteins were used at 20 and 200 nM in H1 kinase assays (Figure 3). GST-p21 is somewhat less potent than untagged p21 in these assays; even at 200 nM, 3–5% activity remained. Removal of residues 81–164 had little effect on inhibitory activity, suggesting that residues 1–80 are sufficient for inhibition. In contrast, residues 1–60 lacked appreciable inhibitory activity at 200 nM. This is consistent with the observation that

residues 1–80 display ~44% sequence identity with p27 in a domain that has been shown to possess inhibitory activity when expressed alone (Polyak *et al.*, 1994).

Overproduction of p21 Leads to G1 Arrest

Previously, we demonstrated that p21 overexpression blocks the cell cycle outside of S-phase in normal diploid fibroblasts (Harper *et al.*, 1993), although these experiments did not identify the cell cycle position where arrest occurs. The data presented above predicts that p21 would arrest in G1. To investigate whether p21 arrests at a unique position in the cell cycle, we utilized a transient transfection assay (van der Heuvel and Harlow, 1993; Zhu *et al.*, 1993). An expression plasmid for p21, pCMV-p21, was transfected into VA13 or SAOS-2 cells along with a plasmid expressing the cell surface marker CD20, and after 48 h the DNA content of cells expressing high levels of CD20 was measured by flow cytometry (Figure 4). Previously, it was shown by using this approach that p27 (Toyoshima and Hunter, 1994) and dominant negative forms of Cdk2 and Cdk3 can block cells in G1 (van der Heuvel and Harlow, 1993), thus the Cdk2 dominant negative plasmid (Cdk2-DN) was used here as a positive control for G1 arrest. Both of the cell lines employed (VA13 [WI38 normal diploid fibroblast transformed with SV40] and SAOS-2) have nonfunctional p53 and Rb proteins and low to undetectable levels of p21.

VA13 cells transfected with vector and pCMV-CD20 alone contain 38% G1 phase cells (Figure 4). CD20 positive cells from a p21 cotransfection display a dramatic G1 accumulation, with 79% of the transfected population having a G1 DNA content. Both S and G2/M phases are concomitantly reduced. As expected, a similar G1 arrest was found with Cdk2-DN (Figure 4) in these cells. SAOS-2 cells have been extensively characterized with respect to cell cycle arrest by both Cdk2-DN and Rb, both of which lead to predominantly a G1 arrest (Hinds *et al.*, 1992; van der Heuvel and Harlow, 1993). SAOS-2 cells also accumulate in G1 when transfected with p21 (45% vs. 68% in G1). The overall distribution of cell cycle phases in p21 expressing cells is quite similar to that resulting from expression of Cdk2-DN or Rb (Figure 4), consistent with the fact that p21 preferentially inhibits Cdks involved in the G1/S transition. These data indicate that p21 alone can function to arrest the cell cycle in G1 and does not absolutely require other p53-regulated proteins or Rb. Furthermore, these data are consistent with the idea that p21 may be the primary mediator of G1 arrest induced by the p53 checkpoint pathway (Dulic *et al.*, 1994; El-Deiry *et al.*, 1993, 1994).

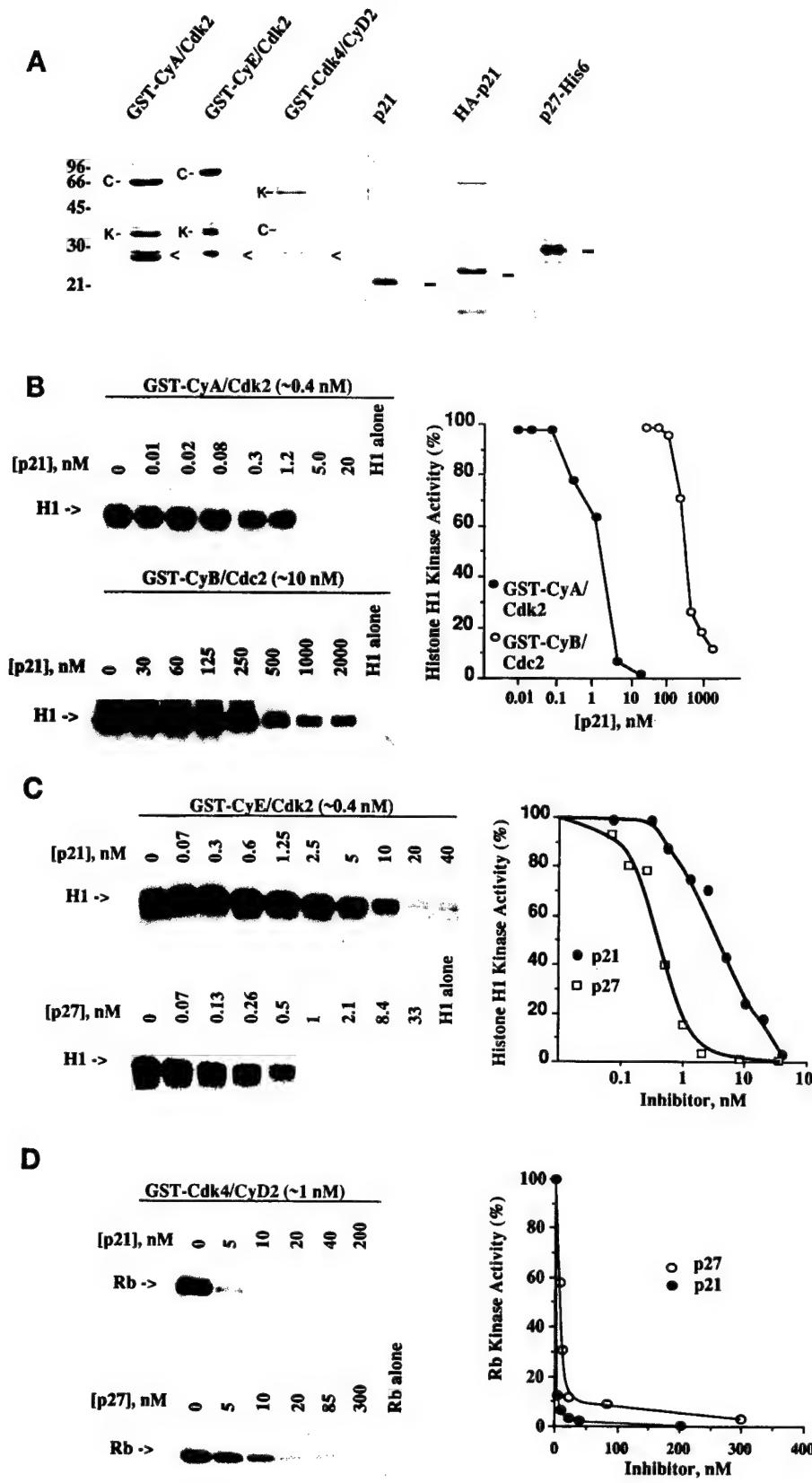


Figure 2. Determination of the K_i values for inhibition of Cdk5 by p21 and p27. (A) SDS-PAGE analysis of purified proteins. Proteins were purified as described under MATERIALS AND METHODS. The arrowheads indicate the position of insect cell glutathione binding proteins, K- indicates the position of the designated kinase subunit, C- indicates the position of the designated cyclin subunit, and the bar indicates the designated inhibitor protein. (B) Inhibition of purified GST-cyclin A/Cdk2 (~0.4 nM) and GST-cyclin B/Cdc2 (~10 nM) by p21. Assays were carried out at $[E] < K_i$ for 1 h (Cdk2) or 30 min (Cdc2) at 37°C. Reaction mixtures were analyzed by SDS-PAGE and autoradiography. (C) Inhibition of GST-CyE/Cdk2 by p21 and p27. Assays were performed with purified GST-cyclin E/Cdk2 (0.5 nM) using the indicated quantity of p21 or p27 purified from *E. coli*. One-half of reaction products were used for filter binding quantitation (see MATERIALS AND METHODS) and the remainder was analyzed by SDS-PAGE. (D) Inhibition of GST-Cdk4/cyclin D2 by p21 and p27. Assays were performed using purified GST-Cdk4/cyclin D2 (~1 nM) and soluble Rb (2 μ M) as substrate.

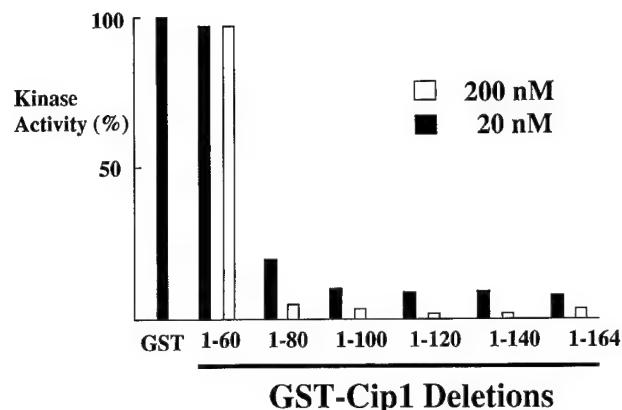


Figure 3. Deletion analysis of p21. The indicated C-terminal deletions of p21 were purified from *E. coli* as GST-fusion proteins and assayed for inhibition of Cdk2/cyclin A using histone H1 as substrate. Activities were quantitated by filter binding (solid symbol, 20 nM GST-fusion; open symbol, 200 nM GST-fusion).

Association of p21 and p27 with Cdks Is Greatly Enhanced by Cyclin

Cdks exist in both monomeric and cyclin-bound forms. High concentrations of immobilized GST-p21 can bind low but detectable amounts of in vitro-translated Cdk2 in the absence of added cyclin (Harper *et al.*, 1993). However, the high affinity observed in inhibition assays using Cdk/cyclin complexes led us to examine whether the affinity of p21 for Cdks is altered by association with cyclins. [³⁵S]Methionine-labeled Cdk2HA and p21 sf9 lysates were mixed under conditions where p21 is limiting and increasing amounts of GST-cyclin E lysate was added (Figure 5A). The total quantity of labeled sf9 cell extracts was kept constant. Cdk2HA and associated proteins were then isolated using anti-HA antibodies and analyzed by SDS-PAGE. In the absence of added cyclin, p21 was not detected in association with Cdk2HA. However, the addition of increasing amounts of GST-cyclin E led to accumulation of p21 in Cdk2HA immune complexes (Figure 5A). These data indicate that cyclin substantially increases the affinity of p21 for complexes containing Cdk2. Analogous experiments with Cdk4/cyclin D2 (Figure 5B), Cdk2/cyclin A (Figure 5C), and Cdk6/cyclin D2 (our unpublished results) indicate that cyclin-dependent association with Cdks is a general property of p21 and does not strictly depend on the identity of the cyclin subunit. In addition, at the low concentrations of Cdk2 and cyclin A used in these mixing experiments, the data in Figure 5C indicates that p21 stabilizes the cyclin A/Cdk2 complex. In contrast with these cyclin/Cdk complexes, p21 does not associate efficiently with Cdk7/cyclin H (Figure 5D).

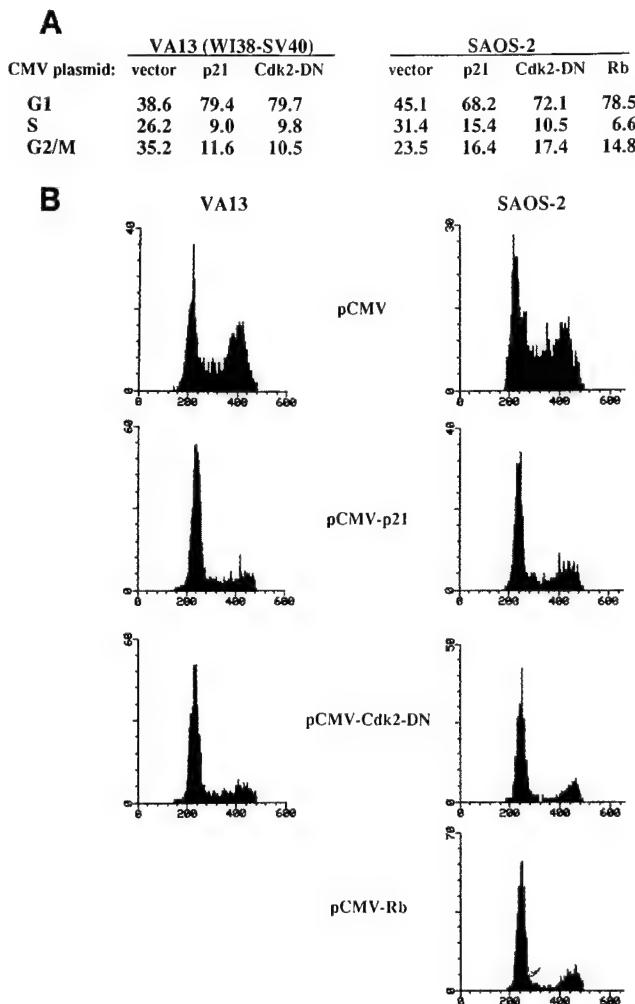


Figure 4. Induction of G1 arrest by transient transfection of pCMV-p21. VA13 fibroblasts or SAOS-2 cells were cotransfected with 28 μ g of either pCMV, pCMV-p21, pCMV-Cdk2-DN, or pCMV-Rb and 2 μ g of pCMV-CD20, and analyzed for DNA content by FACS as described under MATERIALS AND METHODS. In the histograms shown, DNA content is shown on the abscissa and cell number on the axis. The percentages of cells in G1, S, and G2/M are provided in the table.

Like p21, association of p27 with cyclin A/Cdk2 and cyclin D2/Cdk4 is cyclin dependent (Figure 5E). This together with the sequence similarity between p21 and p27 suggests that other features of their inhibitory mechanism may be conserved.

Enhanced association in the presence of cyclin could potentially reflect a strong and direct interaction of these inhibitors with cyclin that is independent of the kinase subunit. To test this possibility, we examined the interaction of 300 nM p21 or p27 with 80 nM cyclin A using immunoprecipitation of proteins purified to homogeneity from *E. coli*, followed by immunoblotting of immune complexes to detect any associated p21 or p27 (see MATERIALS AND METHODS). The

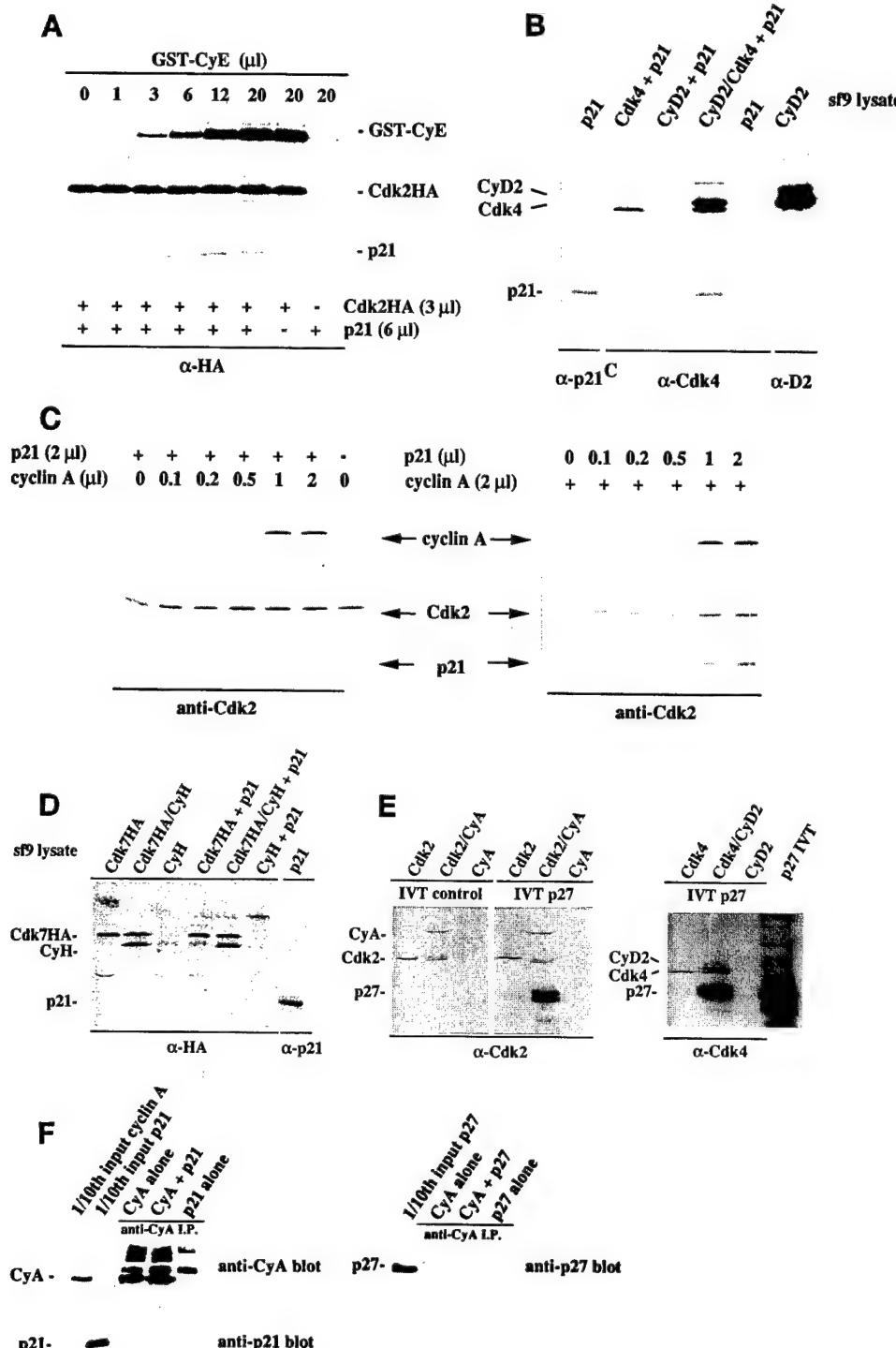


Figure 5. Association of Cdkks with p21 and p27 is enhanced by cyclin. (A) A constant amount of [³⁵S]methionine-labeled sf9 cell lysate containing Cdk2HA was incubated with a constant limiting amount of labeled p21 lysate for 10 min at 4°C. The indicated amount of labeled GST-cyclin E lysate or control lysate was added and anti-HA immune complexes were collected as described under MATERIALS AND METHODS. Samples were fractionated by SDS-PAGE before autoradiography. (B) Cyclin-dependent association of p21 with Cdk4/cyclin D2. Labeled p21 lysate was mixed with either Cdk4 lysate, Cdk4/cyclin D2 lysate, or D2 lysate and the indicated immune complexes were collected as described under MATERIALS AND METHODS. As a control, anti-p21^C directed against the C-terminus of p21 and anti-cyclin D2 immune complexes were also prepared. (C) Cyclin-dependent association of p21 with Cdk2/cyclin A. A constant amount of Cdk2 lysate was mixed with either a constant amount of p21 lysate or cyclin A lysate and increasing amounts of either cyclin A or p21 lysates were added as indicated. Anti-Cdk2 immune complexes were isolated and bound proteins were analyzed by SDS-PAGE and autoradiography. (D) The indicated sf9 lysates containing Cdk7HA and/or cyclin H proteins were mixed with control lysates or lysates containing p21 and then immunoprecipitated with anti-HA or anti-p21 antibodies before SDS-PAGE. (E) The indicated sf9 cell lysates were incubated with 4 μl of in vitro-translated p27 and the indicated immune complexes were collected. Samples were analyzed by SDS-PAGE and autoradiography. (F) The indicated proteins purified from *E. coli* (300 nM p21 or p27 and 80 nM cyclin A) were mixed in a final vol of 150 μl of NETN and subjected to immunoprecipitation using anti-cyclin A antibodies as described under MATERIALS AND METHODS. Immune complexes were immunoblotted using the indicated antibodies. Detection was accomplished using ECL detection (Amersham, Arlington Heights, IL).

cyclin A preparation employed can stoichiometrically activate Cdk2 (Connell-Crowley *et al.*, 1993) and this complex is inhibited by p21 with an apparent K_i value similar to that obtained with insect cell-derived cyclin

A/Cdk2 (Harper *et al.*, 1993 and our unpublished data). In addition, the cyclin A antibodies used for the immunoprecipitation have been previously shown to immunoprecipitate complexes containing p21 (Harper

et al., 1993). As shown in Figure 5F, neither p21 nor p27 associate tightly with cyclin A under washing conditions identical to those employed in Figure 5, A-E. Although less than 1% of the input p21 was found to be associated with cyclin A, no p27 was detected in the cyclin A immune complex. The conditions of this experiment were designed such that less than 1% of the input p21 and p27 could be readily detected. These data indicate that the cyclin-dependent association of p21 and p27 with cyclin A/Cdk2 is not due simply to a direct avid interaction of the inhibitor with the cyclin that is independent of the kinase subunit.

p21 Is Associated with the Majority of Active Cdk2 in Normal Fibroblasts

Although it is clear that p21 can act as an inducible inhibitor of S phase entry, it is not clear what role it plays in actively growing cells. One possibility is that it functions as a buffer to determine the level of active kinase required for passage through particular cell cycle transitions. To understand the physiological significance of this buffer, it is important to know what fraction of kinase is associated with p21 *in vivo*. Quantitative immunoblot analysis of WI38 fibroblast extracts was performed using *E. coli* derived HA-p21 and HA-Cdk2 as standards (see MATERIALS AND METHODS). As shown in Figure 6A, p21 levels are two- to fourfold higher than the total Cdk2 protein, as determined by the relative immunoblot signals of HA-p21 and HA-Cdk2 determined in parallel. This p21 is presumably distributed among multiple Cdks (Xiong *et al.*, 1992; Zhang *et al.*, 1993).

To determine what fraction of Cdk2 in normal diploid fibroblasts is bound to p21, we depleted p21 from [³⁵S]methionine-labeled WI38 extracts by sequential immunoprecipitations and then determined what fraction of active Cdk2 remained in the depleted extract using histone H1 as substrate (Figure 6B). As a control for Cdk2 immunoprecipitation and kinase activity, two sequential Cdk2 immune complexes were prepared from an equivalent amount of lysate (Figure 6B, lanes 6 and 7). This experiment revealed the following: 1) the vast majority of active Cdk2 complexes are associated with p21 in normal diploid fibroblast, and 2) p21 immune complexes contain substantial levels of H1 kinase activity. The activity of the Cdk2 immune complexes from the undepleted extract was 40% of that contained in p21 immune complexes. In contrast, the kinase activity of the Cdk2 immunoprecipitates from the p21-depleted extract (Figure 6B, lane 5) was essentially indistinguishable from background activity found in normal mouse serum immune complexes (Figure 6B, lane 1).

The observation that active kinases are present in p21 immunoprecipitations is surprising, given the fact

that p21 inhibits Cdk activity, and was recently reported by Zhang *et al.* (1994). This activity could be due to several possibilities. Modified forms of p21 or Cdk/cyclin complexes could exist that can bind each other but do not inhibit kinase activity. Alternatively, unrelated kinases could be present in these complexes as part of a higher order complex containing p21 and Cdks. Both of these explanations predict that the kinase activity in p21 immune complexes would be resistant to inhibition by exogenous p21; however, because exogenous p21 can block the p21-associated H1 kinase activity (Figure 6B, lanes 8 and 9), both possibilities are eliminated. We also considered the possibility that kinase activity was due to re-equilibration of immune complexes after isolation as a result of changing the buffer composition. As shown in Figure 6C, greater than 93% of the anti-p21-associated kinase activity remained associated with anti-p21 immune complexes under the conditions of the kinase assay, ruling out re-equilibration as an explanation for kinase activity in p21-containing complexes.

An alternative explanation is that p21 can associate with Cdks in a noninhibitory mode. For example, inhibition could require association of multiple p21 molecules, with association of the first molecule being noninhibitory, as suggested recently by experiments utilizing crude insect cell lysates (Zhang *et al.*, 1994). In this scenario, p21 immune complexes from cell extracts would contain a mixture of inhibited and active Cdks, depending upon the relative levels of p21 and cyclin-associated Cdks in the particular extract. To test this hypothesis, the kinase activity of anti-HA immune complexes generated from samples containing a constant amount of Cdk2/cyclin A and varying amounts of HA-p21 was examined (Figure 7, A and B). For these experiments, HA-p21 purified from *E. coli* (Figure 2A) and [³⁵S]methionine-labeled Cdk2/cyclin A, purified to apparent homogeneity from sf9 cells (see MATERIALS AND METHODS), was used. This Cdk2/cyclin A preparation migrated at the expected position for a monomeric complex using gel filtration (Figure 7C). The use of pure proteins avoids potential artifacts that could come into play when crude sf9 extracts are used (Zhang *et al.*, 1994).

As shown in Figure 7A, increasing amounts of Cdk2/cyclin A were immunoprecipitated with increasing amounts of HA-p21. Quantitation of labeled proteins revealed that the maximum amount of Cdk2/cyclin A recovered with anti-HA antibodies was ~60% of that recovered with anti-Cdk2 antibodies. At the lower concentrations of HA-p21 used, substantial histone H1 kinase activity was observed in these immune complexes (Figure 7B), but as the concentration of HA-p21 was increased, the Cdk2 kinase activity diminished sharply. The specific activity of the HA-p21/Cdk2/cyclin A complex associated with the lowest amounts of HA-p21 was at least 20% of that of the

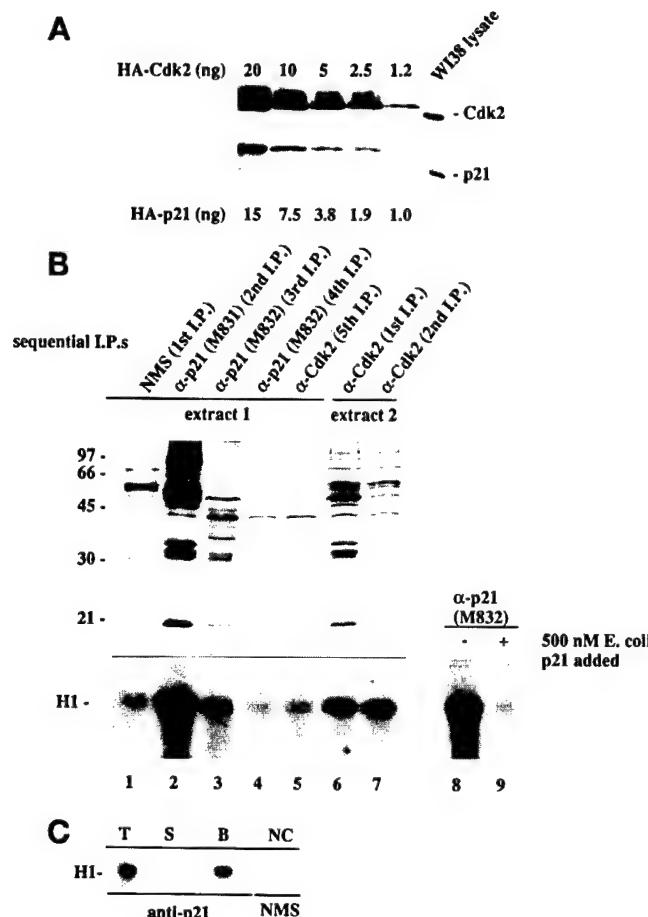


Figure 6. p21 is associated with the majority of active Cdk2 in WI38 fibroblast extracts. (A) Quantitative immunoblot analysis of p21 and Cdk2 in extracts from WI38 cells. A dilution series of the indicated quantities of HA-p21 and HA-Cdk2 (determined as described under MATERIALS AND METHODS) were subjected to immunoblotting along with a 10 μ l aliquot of WI38 cell extract (equivalent to \sim 210,000 cells) and the blot was probed with anti-Cdk2 and anti-p21 antibodies. Detection was accomplished using ECL. Assuming similar transfer efficiency for p21 and Cdk2, the molar ratio of p21 to Cdk2 is 2–4. (B) Extracts from [35 S]methionine-labeled WI38 cells were immunoprecipitated sequentially with either normal mouse sera (NMS), anti-p21, or with anti-Cdk2, as indicated. Extracts corresponding to one 10-cm dish were used for each immunoprecipitation. Twenty percent of each immune complex was used for histone H1 kinase assays (30 min, 37°C) and the remaining immune complex was separated by SDS-PAGE before autoradiography. A third aliquot of unlabeled fibroblasts (extract corresponding to 1/2 of a 10-cm dish) was subjected to immunoprecipitation with anti-p21 and the immune complexes divided equally. To one of the two immune complexes, 500 nM p21 purified from *E. coli* (Figure 2) was added. Histone H1 kinase assays were performed as described above. Histone H1 kinase activities were assessed by SDS-PAGE and autoradiography. (C) The majority of active Cdk2 remains associated with anti-p21 immune complexes during the kinase assay. WI38 lysates (500 μ g protein) were immunoprecipitated with anti-p21 mIgG (in duplicate) or with NMS and immune complexes prepared for kinase assays as described under MATERIALS AND METHODS. One anti-p21 complex and the negative control complex were kept on ice (30 min) while the second anti-p21 complex was incubated at 37°C in the presence of 1 μ M

Cdk2/cyclin A complex, based on the activity of the anti-Cdk2 immune complexes assayed and quantitated in parallel (Figure 7A). Interestingly, at 70 nM HA-p21, approximately 30 nM Cdk2 was precipitated and this complex is essentially inactive. These results rule out bridging molecules as being involved in p21-associated kinase activity and are consistent with the idea that multiple molecules of p21 can associate with Cdk/cyclin complexes (Zhang *et al.*, 1994).

p21 Levels Can Serve to Set the Threshold for Cdk Activity In Vitro

In previous experiments (Figure 7 and Zhang *et al.*, 1994), the activity of p21-associated kinases were examined with increasing levels of p21 and under nonequilibrium conditions (i.e., after immunoprecipitation). An alternative approach for examining p21 function that more closely resembles the situation in cycling cells is to keep p21 levels constant and increase cyclin/Cdk levels under equilibrium conditions. As shown in Figure 8, reaction of histone H1 peptide with increasing levels of Cdk2/cyclin A kinase results in an approximately linear increase in peptide phosphorylation, except at very high kinase concentrations. In contrast, when increasing amounts of pure Cdk2/cyclin A are added to reaction mixtures containing a fixed concentration of pure p21 (with $[I] > K_i$), a threshold effect is observed. At low cyclin/Cdk levels, activity is completely inhibited, whereas at concentrations of kinase exceeding the functional form of the inhibitor, kinase activity increases sharply. These data suggest that when cyclin/Cdk levels are low, compared with the p21 levels, the majority of cyclin/Cdk in complex with p21 is in the inhibited form. The distribution of Cdk complexes in active and inactive forms will depend on the relative abundance of Cdk, cyclins, p21, and other Cdk inhibitors. One other point that can be inferred from the data in Figure 8 is that the slopes of the curves for kinases in the presence of inhibitor are slightly lower than the slope for the free kinase itself. If kinases with a single p21 bound are as active as unbound kinase, the slopes should be the same. This may indicate that the kinase activity of complexes containing one p21 is slightly reduced.

histone H1 but without ATP. After 30 min, the supernatant from the mock kinase reaction was removed to a new tube, the immune complex was reconstituted with kinase buffer, and H1 kinase reactions were performed on all of the immune complexes in the presence of [32 P]ATP (30 min, 37°C). Reactions were analyzed by SDS-PAGE and quantitated by phosphoimager analysis.

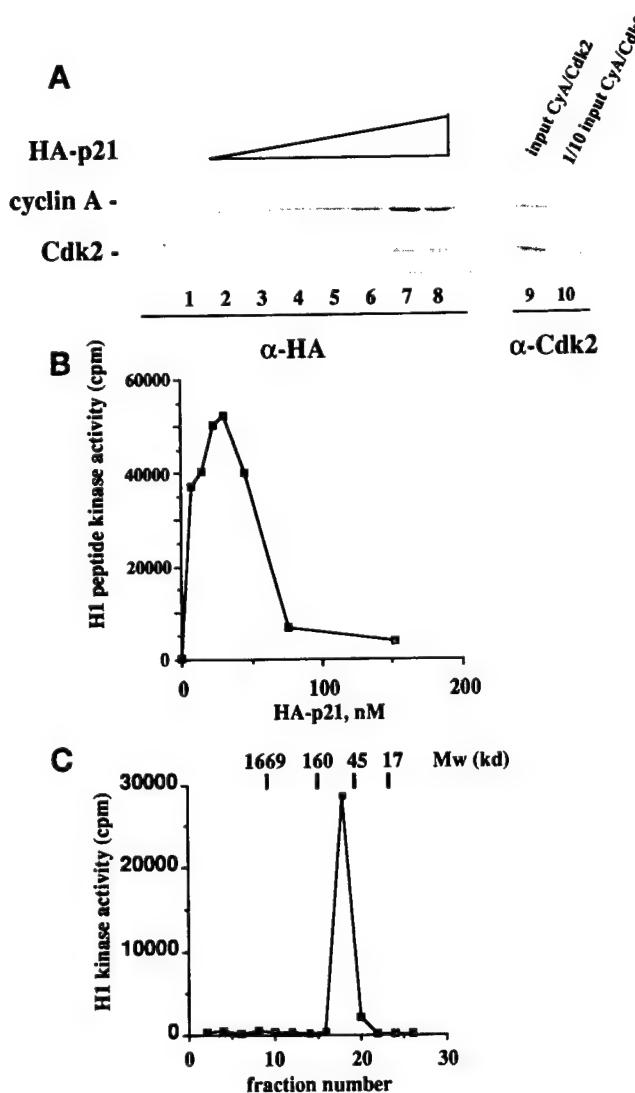


Figure 7. Association of active forms of Cdk2/cyclin A with p21. A constant amount of purified [³⁵S]methionine-labeled cyclin A/Cdk2 (~50 ng Cdk2) was incubated with increasing amounts of HA-p21 purified from *E. coli* (see panel B for concentrations) in a total vol of 30 μ l of EB. After 10 min, 3 μ g of anti-HA antibodies, 10 μ l protein A-Sepharose, and 150 μ l of binding buffer were added before immunoprecipitation. As a control, anti-Cdk2 immunoprecipitations were carried out on ~50 and ~5 ng of cyclin A/Cdk2. Eighty percent of the immune complex was subjected to SDS-PAGE and autoradiography (A) while the remainder was assayed for kinase activity using histone H1 peptide as substrate (B). The activity of the immune complex derived from 5 ng Cdk2 gave 350,000 cpm. Based on the quantitation of ³⁵S-labeled Cdk2 present in the anti-HA and anti-Cdk2 immunoprecipitates using phosphoimager analysis, the maximum specific activity for the anti-HA complex was 20% of that of Cdk2/cyclin A (at 7 nM HA-p21). (C) Cyclin A/Cdk2 migrates as a monomeric complex on a gel filtration column. Approximately 3 μ g of purified cyclin A/Cdk2 (in 0.2 ml column buffer) was chromatographed on a Superose-12 column as described previously (Gu *et al.*, 1993). Two microliter aliquots were assayed for histone H1 peptide activity (1 h, 37°C). The elution positions of molecular weight markers are shown.

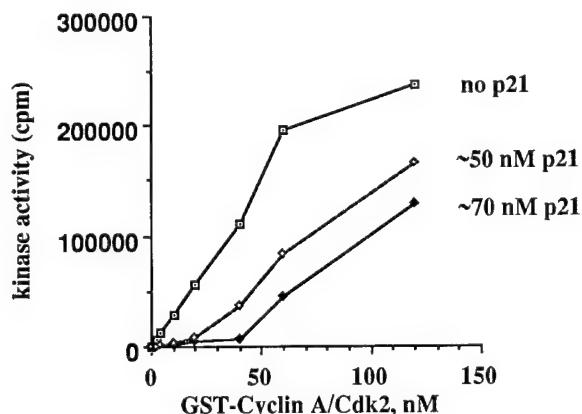


Figure 8. p21 can determine the threshold concentration of Cdk/cyclin complexes necessary for production of active kinase. A constant amount of p21 was mixed with varying quantities of purified GST-cyclin A/Cdk2 in a total vol of 0.02 ml and kinase assays were initiated by addition of histone H1 peptide and ATP. After 15 min at 37°C, reactions were quenched by addition of an equal vol of 50 mM EDTA and activities determined as described under MATERIALS AND METHODS.

DISCUSSION

p21^{Cip1} was originally identified as an inhibitor of Cdk2, Cdk4, and Cdc2 kinase complexes. Here we show that it can inhibit with high affinity all kinases known or suggested to have a direct role in the G1 to S phase transition including Cdk2, Cdk3, Cdk4, and Cdk6 but is a poor inhibitor of other known Cdks. For Cdc2/cyclin B, 400-fold higher p21 levels are required to achieve the same degree of inhibition as with Cdk2 kinases. Although p21 is found to be associated with Cdc2/cyclin B in extracts from normal diploid fibroblasts (Xiong *et al.*, 1993a; Zhang *et al.*, 1994), the stoichiometry and degree of inhibition of this kinase in this context has not been determined. Therefore, it is not known whether the high K_i value reflects poor binding or merely an inability to inhibit once bound. It is possible that other cellular proteins, not present in the *in vitro* setting, could increase the affinity of p21 for Cdc2/cyclin B complexes. In addition, p21 shows no inhibitory activity toward Cdk5/p35 complexes and does not associate with the Cdk-activating kinase Cdk7/cyclin H. Cdk5 was previously detected in extracts from normal diploid fibroblast in association with D-type cyclins and p21; however, Cdk5 is not activated by D-type cyclins *in vitro* or *in vivo* (Xiong *et al.*, 1992; Tsai *et al.*, 1993, 1994; our unpublished results) and the relevance of association of p21 with Cdk5 in fibroblast extracts is unclear at present. If p21 can bind Cdk5/p35, it does not effectively inhibit kinase activity. Taken together, our data indicate that p21 is not a universal inhibitor of Cdks, but shows selectivity for kinases that regulate G1 decisions.

Consistent with its biochemical selectivity, p21^{Cip1} overproduction arrests cells in G1. This property is consistent with its hypothesized role in the mediation of p53's checkpoint function (El-Deiry *et al.*, 1993, 1994; Dulic *et al.*, 1994). In contrast with the Cdk2 dominant negative protein that blocks cells in G1, the Cdc2 dominant negative arrests cells in G2/M (van der Heuvel and Harlow, 1993). This suggests that if p21 overexpression functioned to potently inhibit Cdc2 in vivo, then the G2/M population of cells would have either been maintained or increased in our p21 transfection experiments. p21^{Cip1} has been shown to be capable of blocking the action of PCNA in DNA replication assays *in vitro* (Flores-Rozas *et al.*, 1994; Waga *et al.*, 1994). Interestingly, we do not see a large percentage of cells arresting in S phase. It is possible that the kinetics of this assay are inappropriate to detect such arrest. If lower levels of p21 are required to arrest in G1 than for blocking DNA replication, then the lower level will be reached first, thereby blocking G1 cells from entering S but allowing cells in S to complete S phase before levels of p21 high enough to block DNA replication are achieved. Alternatively, the ability to block DNA replication *in vitro* may not faithfully represent the *in vivo* situation.

Although GST-p21 interacts weakly with Cdks produced by *in vitro* translation (Harper *et al.*, 1993), we have found that the affinity of p21 for Cdks is greatly increased if the Cdk is associated with a cyclin. This is true for all Cdk/cyclin complexes examined and extends to its related inhibitor p27. Although GST-p27 has been reported to associate with D-type and E-type cyclins in reticulocyte lysates (Toyoshima and Hunter, 1994), these results are complicated by the presence of Cdks in reticulocyte lysates, which could potentially mediate the observed interactions. Our results obtained using cyclin and inhibitor proteins purified from *E. coli* indicate that cyclin A does not associate with either p21 or p27 efficiently in the absence of a kinase subunit under conditions where trimeric complexes are readily observed. Thus, if contacts are made with cyclins, they are very weak, and the cyclin dependency would result from integration of weak interactions with surfaces on both cyclins and Cdks. Alternatively, the enhanced affinity for Cdks in the presence of a cyclin could result from conformational changes on the Cdk or cyclin induced upon formation of the cyclin/Cdk complex that favor inhibitor binding.

Cyclin-dependent association has important physiologic consequences for p21^{Cip1} function. For example, if p21^{Cip1} binds monomeric and heteromeric forms of Cdks equally well, when cells in G1 begin to synthesize cyclins, those cyclins could bind either kinase monomers that already have inhibitor bound or free monomers. The inhibitor would reduce a straight percentage of active kinases as they form, dampening the

rise in kinase activity. In contrast, if p21^{Cip1} preferentially associates with cyclin/Cdk complexes, as cyclins are synthesized they bind Cdks and then are inactivated by p21. Therefore, p21^{Cip1} acts as a buffer, soaking up potentially active kinase. This buffer must be overcome to produce the active kinase needed to catalyze the G1 to S transition. Our *in vitro* experiments (Figure 8) are consistent with the idea that p21 can function as a titratable buffer and can act to set the cyclin threshold necessary for cell cycle progression.

Our finding that virtually all of the active Cdk2 in extracts from growing fibroblasts is complexed with p21^{Cip1} was unexpected. *In vitro* reconstitution experiments utilizing purified proteins indicate that multiple p21 molecules are required for inhibition, as has been suggested recently by experiments performed using crude cell extracts (Zhang *et al.*, 1994). Although in principle, the stoichiometry of inhibition could be determined using pure proteins, uncertainties in the concentrations of active p21 and Cdk2 caused by differential binding of dyes used for protein quantitation and the population of nonfunctional molecules, do not allow firm conclusions to be made. Although detailed structural studies are likely to be required to determine the true stoichiometry of inhibition, our measurements (Figures 7 and 8) are most consistent with the requirement of two molecules for inhibition. The simplest model for association of p21 with Cdk complexes involves binding at two kinetically independent sites with the noninhibitory site having higher affinity than the inhibitory site. The finding that p21-containing complexes can contain kinase activity argues that binding of p21 to the inhibitory site is not strongly cooperative. We cannot exclude the possibility, however, that the affinity of p21 for the inhibitory site is altered by binding of p21 to the noninhibitory site.

What is the physiological significance of a requirement for multiple molecules in the inhibition process? First, the fact that virtually all of the active Cdk in WI38 cells is already bound to p21 suggests that the p21 buffering system is half titrated. This means that it is maximally sensitive to increases in p21 levels. Thus a twofold or greater increase in the concentration of p21, such as that observed in the presence of DNA damage (El-Deiry *et al.*, 1994), should suffice to completely inhibit Cdk2 kinase activity (see Figure 7). This is based upon the assumption that two and not more molecules are required for inhibition. Second, a requirement for two molecules could sharpen the activation curve once the buffer has been fully titrated, although we did not observe this *in vitro* (Figure 8). Third, under certain conditions p21, and possibly p27, could serve to target active kinases to substrates or effector molecules, as appears to be the case with PCNA and p21.

Many details of the biochemical mechanism of p21 action remain to be elucidated. For example, it is not known whether there are actually two or more binding sites for p21 on the surface of the Cdk/cyclin complex or whether the second p21 molecule associates with itself forming an active dimeric species on the surface of the Cdk/cyclin complex. In this regard, experiments using HA-tagged p21 and untagged p21 have failed to demonstrate direct dimerization of p21 molecules in the absence of a kinase and *E. coli* p21 migrates at the position expected for a monomer on gel filtration (our unpublished data). The sequence identity between p21 and p27 suggests that aspects of their inhibitory mechanisms are conserved. We have shown that both inhibitors display cyclin-dependent binding to Cdks and that like p27, the N-terminal region of p21 is sufficient for inhibition. The potential interaction among inhibitors will be important for understanding the complex interplay between growth regulatory pathways that utilize different inhibitor family members. Our *in vitro* inhibition constants indicate that Cdk4/cyclin D2 is more effectively inhibited by p21 than by p27 and vice versa for Cdk2/cyclin E. This suggests that these inhibitors may initially target different kinases during cyclin accumulation.

In summary, the primary conclusions of this paper are as follows: 1) p21^{Cip1} preferentially inhibits Cdk/cyclin complexes that play a role in the G1/S transition; 2) high levels of p21^{Cip1} can block the cell cycle in G1, consistent with a role in mediating the p53-dependent G1 checkpoint; 3) association of p21^{Cip1} and p27^{Kip1} with Cdks is greatly enhanced by cyclin; 4) multiple p21 molecules are required for inhibition of cyclin/Cdk kinase activity; 5) nearly all active Cdk2 is associated with p21 in extracts from normal diploid fibroblast; and 6) active complexes containing p21, Cdks, and cyclins can be inhibited by exogenous p21. These data are consistent with a model whereby p21^{Cip1} may act as a regulated buffer that sets the threshold cyclin levels necessary to activate the G1 to S phase transition. As cyclin D levels have been shown to be responsive to growth factor concentrations (reviewed in Sherr, 1994), p21^{Cip1} may act to determine the concentration and, perhaps, the identities of growth factors necessary to activate growth of particular cells. Mutations that reduce p21^{Cip1} levels could have significant consequences for endocrine dependent growth such as that observed during development and in certain types of tumors.

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Cyclin Dependent Kinase Inhibitors

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Introduction

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The INK4 family

p21 and the multiple molecule hypothesis

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INTRODUCTION

Progression through the eukaryotic cell cycle requires the action of positive regulatory elements that catalyze passage through particular transitions. Cyclin dependent kinases (CDKs) function in this capacity (Hunter and Pines, 1994; Sherr, 1994). These enzymes are among the most highly regulated enzymes known, reflecting their central importance in cell proliferation. Positive regulation is achieved by both cyclin association and threonine phosphorylation catalyzed by CDK activating kinase (CAK) (reviewed in Morgan, 1995). Negative regulation is achieved through phosphorylation of the catalytic subunit by Wee1/Myt1 like kinases (reviewed in Coleman and Dunphy, 1994) and through association with CDK inhibitory proteins (CKIs; reviewed in Elledge and Harper, 1994; Sherr and Roberts, 1995; Harper and Elledge, 1996).

The discovery of CKIs has provided new paradigms for understanding how extracellular and intracellular signals regulate cell cycle progression. In addition, the finding that some CKIs are tumour suppressors or are regulated by tumour suppressors has provided a direct link between cell cycle control and tumorigenesis. This chapter focuses on CKI function and regulation, with special emphasis on the roles of CKIs in development and cancer as revealed through the analysis of CKI deficient mice.

STRUCTURE AND FUNCTION OF CKIs

The CIP/KIP Family

Two classes of structurally distinct CKIs have been identified in mammals: the CIP/KIP class and the INK4 class. The CIP/KIP class is typified by p21^{CIP1/WAF1}, the first mammalian CKI to be identified (El-Deiry *et al*, 1993; Harper *et al*, 1993; Xiong *et al*, 1993a). This CKI and its family members p27^{KIP1} (Polyak *et al*, 1994; Toyoshima and Hunter, 1994) and p57^{KIP2} (Lee *et al*, 1995; Matsuoka *et al*, 1995) are modular in structure and are sometimes referred to as dual specificity inhibitors (Harper and Elledge, 1996). Although this class of CKI is commonly referred to as "universal" CDK inhibitors (Xiong *et al*, 1993a), this description is somewhat misleading. For example, p21 is a potent inhibitor of the G₁ CDKs (CDK2, CDK3, CDK4 and CDK6), with K_i values from 0.5 to 5 nM, but is a poor inhibitor of CDC2 (K_i ~400 nM) and does not associate with CDK7/cyclin H or CDK5/p35 (Harper *et al*, 1995). Thus, overexpression of these inhibitors leads to arrest in G₁ (Polyak *et al*, 1994; Toyoshima and Hunter, 1994; Harper *et al*, 1995; Lee *et al*, 1995; Matsuoka *et al*, 1995). Inhibition is mediated by a conserved N-terminal approximately 60 residue domain (Harper *et al*, 1995; Luo *et al*, 1995). The interaction with CDK/cyclin complexes is likely to involve extensive regions of p21, and although results differ depending on the experimental system used, most of the available data indicate that high affinity association of p21 with CDKs requires cyclin association (Zhang *et al*, 1994; Goubin and Ducommun, 1995; Hall *et al*, 1995; Harper *et al*, 1995; Zhu *et al*, 1995). It is likely that p21 contacts both the cyclin and the kinase subunits. Alanine scanning mutagenesis of clusters of charged residues has revealed several regions of sequence that when mutated reduce, but do not abolish, association with CDK2 (Goubin and Ducommun, 1995). Only one point mutation (D52A) reduced association with CDK2 dramatically (~33-fold). This residue is in a relatively conserved region of the protein but is not conserved in mouse p57. The N-terminal domain contains a short conserved sequence motif that is also found in the CDK binding domain of an otherwise unrelated negative cell cycle regulator p107, a relative of RB (Zhu *et al*, 1995). When p107 is in complexes with CDK2/cyclin A, p107 becomes the exclusive substrate of the kinase and blocks phosphorylation of exogenous substrates that do not form tight complexes with the kinase (eg RB). Thus, in particular situations, p107 may behave as a CDK inhibitor. The finding that induction of p21 can lead to formation of cyclin A/CDK2/p21 complexes at the expense of p107 indicates that they share common or overlapping binding sites on the kinase (Zhu *et al*, 1995).

In addition, each inhibitor contains a C-terminal domain that is not required for inhibition but may serve as a binding site for other proteins. In fibroblasts, p21 participates in quaternary complexes containing a CDK, a cyclin and proliferating cell nuclear antigen (PCNA) (Xiong *et al*, 1993b). PCNA functions as a processivity factor for DNA polymerase δ and is required for DNA replication. Association of PCNA with these complexes is mediated

through an approximately 10 aminoacid stretch at the C-terminus of p21 (Goubin and Ducommun, 1995; Warbrick *et al*, 1995). Although the biological significance of this association is not well understood at present, association of p21 with PCNA blocks polymerase δ dependent DNA replication in vitro (Flores-Rozas *et al*, 1994; Waga *et al*, 1994). Contradictory reports have appeared on the effects of p21 on excision repair. Whereas Li *et al* (1994) and Shivji *et al* (1994) have found no substantial effect of p21 in excision repair under conditions where DNA replication by polymerase δ is largely inhibited, Pan *et al* (1995) reported that p21 blocks nucleotide excision repair of DNA damaged by either ultraviolet radiation or alkylating agents and that this inhibition is reversible by PCNA. Effects on PCNA in vitro do not appear strictly to require N-terminal and central domains of p21, since a PCNA binding domain peptide is sufficient to inhibit DNA replication in vitro (Warbrick *et al*, 1995). An open question concerns the role of p21-PCNA association in the regulation of DNA replication in the normal cell cycle and during checkpoint activation. Although transient transfection of plasmids expressing the PCNA interaction domain of p21 can reduce the rate of passage through S phase (Luo *et al*, 1995), it is not clear whether the p21-PCNA interaction normally plays an essential part in checkpoint control. This question could be addressed through an analysis of cells containing inactivating mutations in the CDK binding domain of p21. In contrast to p21, p27 and p57 do not associate with PCNA. However, these two inhibitors do contain homologous C-terminal domains called the QT domain (Matsuoka *et al*, 1995). This domain may mediate interactions with other proteins.

Two p21 related CKIs have been identified in *Xenopus*, p28^{Kix1} or p27^{Xic1}, which appear to be hybrids between CIP and KIP family members (Su *et al*, 1995; Shou and Dunphy, 1996). Although the C-terminal domains contain sequences with similarity to the QT domains of KIP1 and KIP2, these proteins can associate with PCNA, albeit much more weakly than p21. The three residues that contribute the most to PCNA binding for p21 (Warbrick *et al*, 1995) are not conserved in the *Xenopus* homologues, suggesting that there is an additional motif that can selectively recognize PCNA.

The INK4 Family

The INK4 class of CKIs is composed of p16, p15, p18 and p19 (Serrano *et al*, 1993; Guan *et al*, 1994; Hannon and Beach, 1994; Jen *et al*, 1994; Chan *et al*, 1995; Hirai *et al*, 1995). Unlike the CIP/KIP family, these inhibitors are highly selective for CDK4 and CDK6. They are composed almost entirely of ankyrin motifs. In transformed cells lacking functional RB, p16 levels are increased, and in this situation, p16 is found in complexes with CDK4 and CDK6 at the expense of D type cyclins (Serrano *et al*, 1993; Xiong *et al*, 1993b; Bates *et al*, 1994; Tam *et al*, 1994; Parry *et al*, 1995). This led to the proposal that p16 can displace cyclin D from the CDK (Serrano *et al*, 1993; Guan *et al*, 1994). However, direct displacement of cyclin D by an INK4 homologue has thus far not

been demonstrated (Guan *et al*, 1994; Hall *et al*, 1995; Hirai *et al*, 1995). Addition of INK4 to CDK4/cyclin D complexes leads to rapid inhibition, and studies with p19 have demonstrated that this INK4 homologue can associate with inhibited CDK4 complexes containing cyclin D (Hirai *et al*, 1995). Together, these data indicate that INK4 homologues can associate with both cyclin associated and monomeric CDK4/6 and suggest that the molecular contacts may not be identical in the two complexes. Binding of INK4 with monomeric CDK4/6 would give a heterodimeric complex that cannot associate productively with cyclin D. Any trimeric INK4/CDK4/cyclin D complexes formed could be readily converted to the INK4/CDK complex through cyclin turnover, since it is known that D type cyclins have short half lives compared with CDK4. The development of an inducible system that allows the analysis of INK4/CDK complexes immediately after induction, before secondary events such as cyclin turnover, may help clarify the initial mechanisms of inhibition by INK4 family members.

Currently, the biochemical basis of CDK4 inhibition on p16 is not well understood. However, a p16 derived synthetic peptide corresponding to aminoacids 84-103 is sufficient to bind and inhibit CDK4 *in vitro* and can block cell cycle progression in tissue culture cells (Fahraeus *et al*, 1996). Although this peptide has the properties of p16, its inhibitory potency is about 15 000 times lower than full length p16, which has K_i values in the nanomolar range. This suggests that other regions of the protein may be involved in contacts that enhance the affinity. Several p16 mutants that lose their ability to inhibit CDK4 and block cell cycle progression map to the region corresponding to the peptide (see Fahraeus *et al*, 1996). One of these mutants (G101W), found in patients with familial melanoma, displays temperature sensitive binding to CDK4, and its ability to block cell proliferation is temperature dependent (Parry and Peters, 1996). An additional melanoma mutant (V126D) displays a similar phenotype (Parry and Peters, 1996). It is not yet clear whether this single p16 derived peptide is sufficient both to inhibit the activity of a preformed CDK4/cyclin D complex and to block association of CDK4 with cyclin D.

p21 AND THE MULTIPLE MOLECULE HYPOTHESIS

A curious feature of p21 is that it can be found in association with both active and inactive CDK complexes (Zhang *et al*, 1994; Harper *et al*, 1995). For example, anti-p21 immune complexes from diploid fibroblasts contain histone H1 kinase activity comparable to that found in CDK2 immune complexes (Zhang *et al*, 1994). In addition, most of the active CDK2 in these cells is associated with p21 (Harper *et al*, 1995). However, if additional p21 is added to these immune complexes, the kinase activity is completely inhibited. These and other data have led to the proposal that a single molecule of p21 can associate with the cyclin/kinase complex but that multiple inhibitor molecules

(most likely two) are required to inhibit CDKs. In vitro reconstitution experiments are consistent with this idea but suggest that the p21 containing complexes may have reduced specific activity at least towards some substrates (Harper *et al.*, 1995). The ability of CKI bound kinase complexes to phosphorylate substrates is potentially important, since it might allow the CKI to function in substrate targeting, perhaps through their C-terminal domains. In addition, this model is now frequently used to explain how small changes in p21 levels can dramatically alter CDK activity and cell cycle progression.

Although it is clear that p21 containing CDK complexes can phosphorylate histone H1 in vitro, it is not clear at present whether such complexes are active on their physiological substrates in vivo and if so what their specific activities are. One potential model is that p21 interacts with multiple domains of the cyclin/CDK complex, and in vitro, the CDK binding domain can "breathe" sufficiently to allow for substrate entry and catalysis, perhaps at reduced rates. To date, only histone H1 (Zhang *et al.*, 1994; Harper *et al.*, 1995) and a histone H1 derived peptide (Harper *et al.*, 1995) have been used as substrate in these experiments, and it is conceivable that their molecular properties allow them to be phosphorylated by CDKs containing a single p21 molecule. The extent of phosphorylation may depend on the relative binding constants for histone H1 and the p21/CDK inhibitory interaction. It is conceivable that the affinity of critical cellular targets is not sufficient to compete for p21 in vivo. It is also possible that proteins such as p107 that bind directly to cyclin through a region that overlaps with the p21 binding site on cyclin (Zhu *et al.*, 1995) would not be efficiently phosphorylated by CDK/cyclin complexes containing a single p21 molecule. Therefore, the ability of p21-kinase complexes to be functional is likely to be substrate dependent. In addition, it is not clear whether all CDK/cyclin complexes that associate with these inhibitors behave in the same way, since this phenomenon has been tested only with CDK2/cyclin A. Furthermore, it is not clear to what extent p27 and p57 display this property. Whereas p57 immune complexes from HeLa cells contain histone H1 kinase activity (Harper JW and Elledge SJ, unpublished), activity in p27 immune complexes has not been reported. An analysis of the activities of multiple CDK/cyclin complexes with multiple target substrates will need to be performed if we are to understand the *in vivo* importance of this idiosyncrasy of p21 family members. A structure of the CDK/cyclin/CKI complex will no doubt prove to be critical in understanding the complex mechanism of inhibition displayed by this class of CKIs and may suggest ways to test the relevance of this feature *in vivo*.

REGULATION OF CKI ABUNDANCE THROUGH ANTIMITOGENIC PATHWAYS *IN VIVO* AND *IN VITRO*

When p21 was first identified, it was envisioned that CDK inhibitors would be involved in a variety of regulatory decisions that reflect their versatility. Such

decisions include checkpoint function, cell cycle timing and terminal cell cycle arrest during development. Currently, there is evidence for the involvement of one or more of the known CKIs in these processes, although it is not at all clear in many instances how the temporal and spatial expression of CKIs is controlled. One theme that is emerging is that CKI expression is highly cell type specific *in vivo*. Analysis of p21 and p57 expression during embryonic development and in adult tissues indicates that these two inhibitors are in general expressed in a non-overlapping pattern (El-Deiry *et al.*, 1995; Matsuoka *et al.*, 1995; Parker *et al.*, 1995). The tissue of exception is muscle, where both of these CKIs are expressed (Matsuoka *et al.*, 1995; Parker *et al.*, 1995). The highest levels of expression are found in terminally differentiated cells (El-Deiry *et al.*, 1995; Parker *et al.*, 1995), suggesting that CKIs contribute to cell cycle arrest during development.

CKIs, in particular p21, are induced in several cell types undergoing differentiation in culture (Jiang *et al.*, 1994; Steinman *et al.*, 1994; Halevy *et al.*, 1995; Parker *et al.*, 1995; Liu *et al.*, 1996). However, it is unclear whether CKI expression causes terminal differentiation or is a consequence of it. For example, p21 is induced in response to expression of MyoD (Halevy *et al.*, 1995; Parker *et al.*, 1995), a transcription factor that is sufficient to induce the muscle differentiation pathway and is known to cause cell cycle arrest. However, p21 is not required for cell cycle arrest or differentiation in this system, since mice lacking p21 do not have an obvious defect in muscle development (Deng *et al.*, 1995). It is possible that p21 function is redundant with another cell cycle regulator in muscle. There is also evidence in other systems that CKIs can actually promote particular differentiation events. p21 is induced directly by the vitamin D receptor (Liu *et al.*, 1996). Overexpression of p21 (or p27) in the absence of vitamin D in these cells leads to both cell cycle arrest and expression of differentiation markers (Liu *et al.*, 1996). It is currently unclear whether induction of differentiation markers is simply dependent on cell cycle arrest in G₁ in this particular system.

Although little is known about the patterns of expression and regulation of other CKIs *in vivo*, tissue culture experiments, particularly with p27, are beginning to reveal other pathways that regulate CKI abundance. One theme to emerge from these studies is that p27^{KIP1} is frequently induced upon cell cycle exit in response to mitogen deprivation, antimitogenic signals or contact inhibition, and its levels are reduced when cells are stimulated to enter the cycle (Nourse *et al.*, 1994; Poon *et al.*, 1995; Coats *et al.*, 1996; Hengst and Reed, 1996; Winston *et al.*, 1996). Increased levels of p27^{KIP1} are thought to constitute a barrier to CDK activation that must be overcome during mitogen induced cell cycle progression. One mechanism by which this is achieved is the downregulation of p27^{KIP1} protein levels after growth factor stimulation. Furthermore, antisense inhibition of p27^{KIP1} expression in cycling cells prevents cell cycle withdrawal in response to serum deprivation and allows mitogen independent initiation of DNA replication (Coats *et al.*, 1996). Thus, a major function of extracellular growth promoting agents is the elimination of

functional p27^{KIP1}. In accord with this hypothesis, antiproliferative factors such as rapamycin antagonize the growth factor dependent decrease in p27^{KIP1}, thereby inhibiting S phase entry (Nourse *et al.*, 1994). It should be noted, however, that large alterations in p27^{KIP1} abundance are not universally observed. In cycling Swiss 3T3 cells, p27 is present at low levels relative to its targets and approximately doubles in quiescent cells, still far too low to account fully for CDK inhibition (Poon *et al.*, 1995). It is not clear whether these cells have lost the ability to induce p27^{KIP1} dramatically during quiescence, as observed with human diploid fibroblasts (Hengst and Reed, 1996), or whether another mechanism such as reduction in cyclin expression is the critical event in cell cycle arrest.

For some cell types, signals leading to reduced p27^{KIP1} levels can be distinguished from signals leading to increased cyclin/CDK accumulation. In both Balb/c 3T3 fibroblasts and human T lymphocytes, transition from quiescence into S phase is dependent on the sequential and synergistic action of at least two distinct mitogenic activities. CDK complexes assemble in response to the initial signal that promotes cell cycle entry but is not sufficient for passage through the restriction point. In the absence of the second signal, these complexes are inactive and are associated with p27^{KIP1} (see Firpo *et al.*, 1994; Winston *et al.*, 1996). Exposure to a full complement of mitogenic factors results in the reduction of p27 protein levels, a decrease in the level of Kip1 associated with the CDK holoenzyme and subsequent activation of the kinase.

What controls the levels of p27^{KIP1}? Unlike p21^{CIP1}, transcriptional induction does not appear to be a predominant mechanism for regulating p27^{KIP1} levels. *KIP1* mRNA levels are fairly constant during both cell cycle progression and cell cycle exit, indicating a posttranscriptional control of KIP1 accumulation (Hengst and Reed, 1996). Two distinct mechanisms are at play. Firstly, growth arrest leads to increased translation of KIP1 message (Hengst and Reed, 1996). Secondly, p27 half life is increased, compared with that in asynchronously proliferating fibroblasts (Pagano *et al.*, 1995). In some cell types, p27^{KIP1} levels fluctuate during the cell cycle, being high in G₁/S and low during G₂/M (Hengst and Reed, 1996). Cell cycle dependent alterations in half life may contribute to the periodic decrease in KIP1 protein levels. Clues as to how p27^{KIP1} destruction is regulated have come from the finding that p27^{KIP1} is ubiquitinated in a reaction that can be performed in vitro by the Ubc2 and Ubc3 ubiquitin conjugating enzymes, called E2s (Pagano *et al.*, 1995). This modification targets the protein to the proteosome for destruction. Targeted destruction of CKIs may represent a general mechanism for reducing inhibitor levels in response to mitotic signals. This is suggested by the fact that the budding yeast Cdc28p/Clb_p inhibitor p40^{SIC1} is also regulated by ubiquitin mediated degradation (Schwob *et al.*, 1994). In this system, mutational inactivation of Cdc4p, which is thought to function together with the ubiquitin conjugating enzyme E2 (Cdc34p), results in the accumulation of Sic1p and an inability to enter S phase. Deletion of *SIC1* suppresses the S phase arrest

phenotype of the *cdc4-1*, suggesting that Sic1p is an essential target of this pathway. The identification of E3 proteins that recognize and target CKIs for destruction will help to unravel how cell cycle phase or mitogens regulate CKI destruction.

Certain cell types respond to the action of transforming growth factor beta (TGF β) by arresting the cell cycle in G₁, but this arrest can be overcome by the action of the DNA virus oncoprotein E1A. Recent studies have provided a molecular description of the changes in CKI function that occur in response to TGF β (Reynisdottir *et al.*, 1995) as well as the mechanism by which E1A overrides this antimotitic signal (Mal *et al.*, 1996). In cycling mink lung epithelial cells, p27^{KIP1} levels are insufficient to completely inactivate CDK2 and CDK4 complexes required for cell cycle progression. In response to TGF β , p15^{INK4b} is transcriptionally induced (Hannon and Beach, 1994). Through either direct displacement or complex turnover, p15^{INK4b} accumulates on CDK4 at the expense of p27^{KIP1} and cyclin D. p27^{KIP1}, which is liberated from the CDK4 complexes, is then available for inhibition of CDK2/cyclin E complexes (Reynisdottir *et al.*, 1995). The ability of E1A to block the action of TGF β results from two distinct activities (Mal *et al.*, 1996). Firstly, association of E1A with RB abolishes the requirement of active cyclin D/CDK4 complexes for progression through G₁, thus rendering p15^{INK4b} function irrelevant (reviewed in Weinberg, 1995). Secondly, E1A associates with p27, thereby blocking its ability to inhibit cyclin E/CDK2 activity, which is required for S phase entry even in the absence of RB (Mal *et al.*, 1996). Physical inactivation of CKIs may ultimately emerge as a common mechanism regulating cell cycle progression in response to mitogenic agents. Indeed, recent data suggest the existence of a heat labile inhibitor of p21 that is activated through the MYC pathway (Hermeking *et al.*, 1995).

BIOLOGICAL ROLES OF CKIs REVEALED THROUGH CKI DEFICIENT MICE

Although tissue culture systems facilitate the identification of signalling pathways that regulate CKI expression, such systems do not necessarily recreate the complex biological and cellular interactions found *in vivo*. Thus, the true significance of a CKI to control of proliferation and development can only be realized through analysis of CKI deficient animals. In principle, this approach identifies cell types where a particular inhibitor makes a critical contribution to the balance of negative and positive growth control factors that must be maintained for homoeostasis or proper development. Furthermore, this approach will ultimately reveal the cell types that possess redundant or alternative pathways for negative growth control. The results with the three CKI knockout mice generated to date provide a wealth of information about the roles of these genes in cell cycle control, development and tumorigenesis.

p21 and G₁ Checkpoint Function

p21 is transcriptionally regulated by TP53 in response to DNA damage (Dulic *et al*, 1994; El-Diery *et al*, 1994), and it has been suggested in the literature that p21 may mediate some or all of the known functions of TP53 (El-Diery *et al*, 1993, 1994). TP53 is a central factor in the DNA damage response pathway, and this activity is thought to contribute substantially to its tumour suppressor function (reviewed in Bates and Vousden, 1996). Cells lacking TP53 no longer arrest in G₁ in response to γ irradiation or in G₂ in response to activation of the mitotic spindle checkpoint. In addition, some cell types undergo apoptosis when DNA is damaged, and TP53 is required for this process as well. Analysis of p21 deficient cells indicates that p21 is required for complete G₁ arrest in response to DNA damage (Brugarolas *et al*, 1995; Deng *et al*, 1995) but is not required for the spindle checkpoint or for thymocytic apoptosis (Deng *et al*, 1995). One unexpected outcome of this work was the finding that the G₁ checkpoint with γ irradiation was only partially compromised with loss of p21 (Brugarolas *et al*, 1995; Deng *et al*, 1995), but the checkpoint in response to nucleotide pool perturbations with N-(phosphoracetyl)-L-aspartate was fully compromised (Deng *et al*, 1995). This suggests that there is a second checkpoint function that can partially compensate for p21 loss in the presence of some types of damage. In TP53 null cells, both pathways are non-functional. Unlike TP53 deficient mice, p21 deficient mice do not have increased susceptibility to spontaneous tumours (Deng *et al*, 1995). This finding is in keeping with the finding that p21 is infrequently mutated in human tumours (Shiohara *et al*, 1994). However, two loss of function mutations in p21 have been found during an analysis of prostate tumours, indicating that loss of p21 could potentially contribute to tumorigenesis in some cell types (Gao *et al*, 1995). Together, these results indicate that the anti-apoptotic and anti-oncogenic effects of TP53 are complex and involve much more than simply induction of p21. It is possible that TP53's apoptotic function or G₂ checkpoint function are central to its tumour suppressor function, although a contribution of the G₁ checkpoint function cannot be ruled out.

Proliferative Miscues in Mice Lacking p27^{KIP1}

As noted above, alterations in p27 levels or utilization is a frequent consequence of changes in mitogenic signals. Mice lacking p27 display a number of phenotypes which indicate that this CKI has an important role in limiting the extent of cell proliferation but does not generally function in the process of cellular differentiation (Fero *et al*, 1996; Kiyokawa *et al*, 1996). Perhaps the most dramatic phenotype is that p27 deficient mice are substantially larger than wild type mice, indicating a general proliferative advantage in the absence of this CKI. Although all organs generally are larger than normal, organs that normally express the highest levels of p27 (spleen and thymus) are increased to the largest extent. Increased organ size reflects cell number and not cell size. Thus, cell division in these tissues is regulated largely by p27. Differ-

entiation in these tissues appears to be normal. This has been best characterized in the thymus, where a dramatic increase in cell number and organ size had no effect on thymic development and thymocytic differentiation. Moreover, cells from all haemopoietic lineages were normally represented (Fero *et al*, 1996; Kiyokawa *et al*, 1996).

Also intriguing is the finding that *p27* deficient mice develop intermediate lobe pituitary hyperplasia or adenoma, consistent with the increased size of this organ. The absence of focal lesions surrounded by apparently normal tissue suggests that pituitary adenoma occurs with 100% penetrance and indicates that proliferation of melanotrophic cells of the pars intermedia is negatively regulated largely through *p27*. However, malignant pituitary tumours have not yet been observed in these mice. This phenotype is reminiscent of *RB*^{-/+} mice, which have pituitary tumours concomitantly with the loss of the second *RB* gene (reviewed in Williams and Jacks, 1996). However, unlike *RB*^{-/+} mice, *p27* deficient mice do not die at 9 months of age, indicating that the *RB* deficient tumours are much more aggressive. Thus, *RB* loss is not equivalent to loss of *p27*. Although it is conceivable that pituitary pathology may contribute to altered animal size through endocrine abnormalities, alterations in the levels of growth hormone or insulin like growth factor 1 have not been observed in *p27* deficient mice (Fero *et al*, 1996; Kiyokawa *et al*, 1996).

A third phenotype observed in *p27* deficient mice is female infertility. Although ovarian follicles develop, they do not progress to form corpora lutea. Since *p27* levels are relatively high in the corpora lutea of control mice, it is conceivable that follicle maturation by luteinizing hormone requires the action of *p27* (Fero *et al*, 1996; Kiyokawa *et al*, 1996).

Involvement of *p16* in Cancer Predisposition

INK4 homologues function in a pathway that appears to be a primary target for mutations that contribute to transformation. *INK4* homologues specifically block the activity of CDK4/6, which are thought to regulate *RB* inactivation (reviewed in Sherr, 1994; Weinberg, 1995). These kinases are uniquely activated by D type cyclins. Studies carried out largely in fibroblasts indicate that *RB* is the sole essential substrate of D type cyclin kinases. This is based largely on the finding that *INK4* homologues can block cell proliferation in *RB*^{+/+} cells but not *RB*^{-/-} cells (Koh *et al*, 1995; Lukas *et al*, 1995) and that anticyclin D antibodies inhibit S phase entry in *RB*^{+/+} cells (reviewed in Sherr, 1994; Weinberg, 1995). Loss of this pathway can occur in multiple ways. Loss of *p16* (reviewed in Hirama and Koeffler, 1995) or mutation of CDK4 to a form that no longer tightly associates with *INK4* homologues (Wolfel *et al*, 1995; Zuo *et al*, 1996) may lead to unregulated *RB* phosphorylation and cell cycle entry. Alternatively, overexpression of cyclin D or CDK4 may overcome negative growth control by *INK4* (reviewed in Sherr, 1994).

Given that all four *INK4* homologues occupy the same position in this pathway, it is intriguing that *p16^{INK4a}* is the only *INK4* homologue strongly

implicated in human cancer. *INK4a* and its homologue *INK4b* are located near each other at 9p21 (reviewed in Sherr and Roberts, 1995). This region of the genome is frequently mutated in a wide range of human cancers, and a substantial number of alleles, including the *INK4a* and *INK4b* genes, have been found to contain large deletions. However, whereas missense and nonsense mutations are frequent in *INK4a*, they are quite rare in *INK4b* (Jen *et al.*, 1994). Perhaps the clearest evidence that *p16* is a true tumour suppressor comes from the finding that it is mutant in some familial melanomas (Hussussian *et al.*, 1994; Kamb *et al.*, 1994; Walker *et al.*, 1995). In addition, *INK4a* mutations are frequently found in sporadic cancers of the head and neck, pancreas and oesophagus (reviewed in Hirama and Koeffler, 1995). In addition to mutations, there is also evidence that expression of *p16* is blocked in some tumour types through methylation of its promoter (Herman *et al.*, 1995).

A complicating feature of the *INK4a* locus is that it produces two transcripts from distinct promoters that have alternative first exons (*E1 α* and *E1 β*) but share second and third exons (*E2* and *E3*) (Quelle *et al.*, 1995). The *E1 β* -*E2*-*E3* transcript generates a novel protein, *p19^{ARF}*, from a different reading frame than that for *p16* (Quelle *et al.*, 1995). Although *ARF* does not associate with CDKs, it does block cell cycle progression in *G₁* and *G₂* when over-expressed and thus could formally contribute to tumour suppression at the *INK4a* locus. Although a role for *p19^{ARF}* is not ruled out by the mutational data, many of the data are consistent with the primary involvement of *p16*. Approximately 50% of all mutations affect *p16* alone, with the remaining fraction affecting both *p16* and *p19^{ARF}* (see Serrano *et al.*, 1996, and references therein). In addition, mutations found in familial melanoma kindreds are frequently in *E1 α* . Many nonsense mutations specifically affect *p16*, and a number of missense mutations in *p16* have been shown to affect association with CDK4/6 and cell cycle arrest. However, the fact that there are four known missense mutations in *E2* that affect *p19^{ARF}* but not *p16^{INK4a}* leaves open the possibility that *p19^{ARF}* contributes to tumour suppression in some cell types (Quelle *et al.*, 1995), although these alleles have not been shown to be defective in growth suppression. Because of the complexity at this locus and the paucity of data on expression of *INK4a* products using specific probes, we know little about the role of *INK4a* in development, although the identification of humans that are homozygous null for *INK4a* (Gruis *et al.*, 1995) indicates that these genes do not have an essential role in development.

Mice have been generated that lack exons *E2* and *E3* and therefore delete both the *p16* and *p19* proteins (Serrano *et al.*, 1996). As expected, these mice are viable and, consistently with data in human cancers, loss of *INK4a* leads to greatly enhanced tumorigenesis. Somewhat surprising is the tissue spectrum observed for spontaneous and chemically induced tumours. In contrast to humans, where melanoma, pancreatic and oesophageal tumours are frequently observed with *INK4a* mutations, mice develop primarily fibrosarcomas and lymphomas. Although *INK4a* mutations are seen in these tumour types in humans, they are relatively infrequent. This reiterates the differences in tumour

type specificity frequently observed in mice and humans carrying mutations in the same genes (Harlow, 1992). An additional proliferation phenotype is observed in the spleen, where there is some proliferative expansion of the white pulp along with megakaryocytes and lymphoblasts in the red pulp (Serrano *et al*, 1996). These data suggest abnormal extramedullary haemopoiesis, and this becomes more pronounced with age.

QUESTIONS FOR THE FUTURE

Since mammalian CKIs were first identified in late 1993, there has been substantial progress on many fronts, and significant insights into mechanisms of growth control and tumour suppression have been gleaned (El-Deiry *et al*, 1993; Harper *et al*, 1993; Serrano *et al*, 1993; Xiong *et al*, 1993a). However, there are still many fundamental questions that are not understood.

One question involves the roles of INK4 proteins in development. Although some information is available on the patterns of expression of p21 and p57 during development and in adult tissues, we know little about the cell type specificity of INK4 homologues. Such information could help explain why p16, but not other INK4 proteins, functions as a tumour suppressor in many cell types. The question of functional redundancy will eventually be addressed through the generation of multiply mutant mice.

A second area where our understanding is incomplete concerns the signal transduction pathways that regulate the expression of CKIs during development. We know very little about transcriptional control mechanisms. What are the transcription factors and antimitogenic pathways that regulate induction of CKIs in terminally differentiated cells? A related question concerns the mechanisms that cause destruction of inhibitors. How is p27 destruction regulated and how is p27 recognized for destruction? Do other CKIs employ programmed destruction mechanisms? What is the role of the C-terminal domain of KIP proteins in cell cycle arrest pathways and destruction? Identification of proteins that can associate with the QT domain may help to answer these questions.

The third question concerns the function of p57^{KIP2}. Thus far, point mutations in *KIP2* have not been identified in human tumours (Kondo *et al*, 1996). However, p57 is unique among CKIs in that it is imprinted, being selectively expressed from the maternal allele in all tissues examined except the brain (Hatada and Mukai, 1995; Kondo *et al*, 1996; Matsuoka *et al*, 1996). Although the relevance of this form of regulation is not known, it is consistent with the involvement of p57 in two cancer syndromes, Beckwith-Wiedemann syndrome (BWS) and Wilm's tumour II (WT2) (see Matsuoka *et al*, 1995, 1996, and references therein). BWS is characterized by organ overgrowth and cancer predisposition, WT2 by embryonic tumours, particular in the kidney. The *p57* gene is located at 11p15.5 (Matsuoka *et al*, 1995), very near lesions in these two syndromes. Both syndromes indicate that the tumour suppressor gene(s)

effect is imprinted, since deletions at 11p15.5 in WT2 and balanced translocations in BWS uniquely affect the maternal allele. This, coupled with the fact that *p57* is expressed in many of the tissues most affected by these two syndromes (Matsuoka *et al.*, 1995), suggests that loss of *p57* may contribute to these diseases. Generation of *p57* deficient mice should help in understanding the role of *p57* in development and tumorigenesis.

Finally, we need to solve the riddle of why there are multiple members and distinct classes of CKIs. The CIP/KIP family members have evolved to inhibit multiple G₁ CDKs, whereas the INK4 proteins inhibit only D type cyclins. Available data indicate that CDK2 and CDK3 function at steps distinct from those of CDK4 and CDK6. Why is there an inhibitor class selective for the cyclin D/RB pathway when it is clear that CIP/KIP family members are competent to arrest the cell cycle even in the absence of RB? Moreover, why does loss of *p16*, but not *p21* or *p27*, lead to widespread cancer predisposition. Does it simply reflect tissue specificity and functional redundancy or does it indicate an imbalance in kinase activity that is a reflection of the biochemical specificity of the two classes of CKIs?

SUMMARY

Progression through the eukaryotic cell cycle is regulated by the activities of a family of cyclin dependent kinases (CDKs). These kinases are negatively regulated by phosphorylation and by the action of cyclin kinase inhibitors (CKIs). In mammalian cells, two classes of CKIs have been identified, the INK4 class and the CIP/KIP class. These CKIs are versatile negative regulators of CDK function and have potential roles in development, checkpoint control and tumour suppression. Analysis of CKI knockout indicates that although these inhibitors are not generally required for survival, the phenotypes observed span the gamut of what might be expected for loss of a cell cycle inhibitor. This chapter summarizes our current understanding of the roles of CKIs in growth control.

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Altered cell differentiation and proliferation in mice lacking p57^{KIP2} indicates a role in Beckwith-Wiedemann syndrome

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Mice lacking the imprinted Cdk inhibitor p57^{KIP2} have altered cell proliferation and differentiation, leading to abdominal muscle defects; cleft palate; endochondral bone ossification defects with incomplete differentiation of hypertrophic chondrocytes; renal medullary dysplasia; adrenal cortical hyperplasia and cytomegaly; and lens cell hyperproliferation and apoptosis. Many of these phenotypes are also seen in patients with Beckwith-Wiedemann syndrome, a pleiotropic hereditary disorder characterized by overgrowth and predisposition to cancer, suggesting that loss of p57^{KIP2} expression may play a role in the condition.

Cell proliferation in the embryo is controlled by an intricate network of extracellular and intracellular signalling pathways that process growth regulatory signals and integrate them into the basic cell-cycle regulatory machinery through control of cyclin-dependent kinases (CDKs). CDKs are positively regulated by cyclins and negatively regulated by CDK inhibitory proteins, CKIs^{1,2}. Cyclins D and E function in the G1 phase of the cell cycle and phosphorylate and inactivate the tumour-suppressor retinoblastoma (Rb) and the related proteins p107 and p130, which are negative regulators of the E2F transcription factors that facilitate cell-cycle entry³. There are two classes of CKIs in mammals, the p21^{CIP1} and p16^{INK4} families. Members of the family p16^{INK4} bind and inhibit only Cdk4 and Cdk6 kinases. Mice lacking the tumour-suppressor p16^{INK4} show no gross developmental abnormalities but have high rates of spontaneous tumorigenesis⁴. In contrast, p21^{CIP1}, p27^{KIP1} and p57^{KIP2} CKIs inhibit all G1/S phase CDKs^{1,2}. p21^{CIP1} is transcriptionally regulated by the p53 tumour-suppressor in response to DNA damage, and is required for the G1 DNA-damage checkpoint^{1,2}. Although p21^{CIP1} expression during development correlates with terminally differentiating tissues⁵, mice lacking p21^{CIP1} develop normally⁶. p27^{KIP1}-deficient mice are grossly normal developmentally but display several phenotypes that seem to be linked to cell proliferation^{7–9}. They are larger than wild-type mice, have intermediate-lobe pituitary hyperplasia or adenomas, and females are infertile.

The most structurally complex member of the p21^{CIP1} family of CKIs is p57^{KIP2} (refs 10, 11), which resides at 11p15.5 (ref. 10), a site of frequent loss of heterozygosity in several human cancers including those of the breast, bladder, lung, ovary, kidney and testicle. Several types of childhood tumours display a specific loss of maternal 11p15 alleles, suggesting the involvement of genomic imprinting¹². Rearrangements at 11p15 are seen in Beckwith-Wiedemann syndrome (BWS), which is characterized by numerous growth abnormalities, including macroglossia (enlarged tongue), gigantism, enlarged adrenal glands, ear creases, visceromegaly (enlarged organs), omphalocele (umbilical hernia), kidney abnormalities, advanced ageing and thickening of long bones, and a 1,000-fold increase in the risk of childhood tumours¹³, including

Wilms' tumour, adrenocortical carcinoma, and hepatoblastoma. BWS occurs with an incidence of 1 in 13,700 births, and 15% of these are familial with maternal carriers, suggesting a role for genomic imprinting¹⁴. BWS has highly variable penetrance in which patients usually display only a subset of all phenotypes. BWS has a complex pattern of inheritance including uniparental disomy (paternal), paternal trisomy of chromosome 11p15.5, paternal duplication of the 11p15.5 region, translocations involving maternal 11p15.5 and karyotypically normal transmission.

The protein p57^{KIP2} is encoded by a maternally expressed, imprinted gene in both humans¹⁵ and mice¹⁶, and one cluster of BWS translocations¹⁷ is within 80 kilobases of the p57^{KIP2} gene. A recent study reported that two of nine patients with BWS examined¹⁸ were found to be heterozygous for mutations in the p57^{KIP2} gene. However, there was no analysis of how frequently these alterations were observed in an asymptomatic population. Furthermore, only one patient had an apparent null allele that was shown to be maternally inherited. Because these mutations were found at such low frequency, it is possible that other genes might also be altered in these two patients. To assess directly the role of p57^{KIP2} in development, cancer and BWS, we have generated a mouse lacking the p57^{KIP2} gene. These mice have a variety of developmental defects consistent with a causative role for p57^{KIP2} in BWS, and indicate a role for p57^{KIP2} in control of cell proliferation and differentiation.

Targeted disruption of mouse p57^{KIP2} gene

A targeting construct that removed exons 1 and 2 (Fig. 1a) (87% of the p57^{KIP2} coding region) was introduced into AB2.1 embryonic stem cells. G418/gancyclovir-resistant cells were screened for homologous recombination by Southern blot analysis (Fig. 1b). Homologous recombinant cells were injected into blastocysts from C57BL/6 mice, and male chimaeras were mated to C57BL/6 females. Germline transmission was confirmed by Southern blotting (Fig. 1b). F₁ heterozygous animals were back-crossed to C57BL/6 mice to maintain the disrupted allele. To avoid ambiguity, we indicate the parental origin of alleles in heterozygotes by a superscript m for maternal or p for paternal.

The disrupted allele is a null as demonstrated by the absence of p57^{KIP2} mRNA (Fig. 1e) and protein (Fig. 1c,e) in p57^{+/−} or p57^{−/−} animals. p57^{KIP2} is detected in the tectum of brain, kidney, adrenal gland, muscle, lung and cartilage in wild-type embryos. Levels of p21^{CIP1} and p27^{KIP1} were unchanged in tissues from p57^{KIP2} mutants, except in muscle where a slight increase in p27^{KIP1} was detected (Fig. 1c). The p57^{+/−} animals had wild-type expression of p57^{KIP2}.

Generation of p57^{KIP2} mutant mice

Of 32 offspring from p57^{+/−} female to p57^{+/+} male matings, no p57^{+/−} animals were present when genotyped at two weeks of age (Table 1). Of 82 offspring from p57^{+/−} intercrosses, 55% were

p57^{+/+} and 45% were p57^{−/−} (Table 1); no p57^{−/−} animals survived to two weeks of age. Mendelian inheritance predicts a 1:2 ratio for p57^{+/+} to p57^{−/−} animals, indicating that half of the p57^{−/−} animals died before genotyping. We observed dead or dying new-born mice in several litters, and these were found to be mutant. We concluded that p57^{KIP2} is required for postnatal survival in a hybrid C57BL/6-129Sv background. Although lethal in this background, we recently discovered that crossing to the outbred CD1 strain allows some p57^{+/−} animals to survive well beyond day one.

When evaluated between embryonic day (E)18.5 and E20, genotypes were detected at expected Mendelian frequencies (Table 1). However, 10% of mutant embryos were dead, staged from E13 to E16, consistent with the fact that maternal inheritance of the p57^{KIP2}

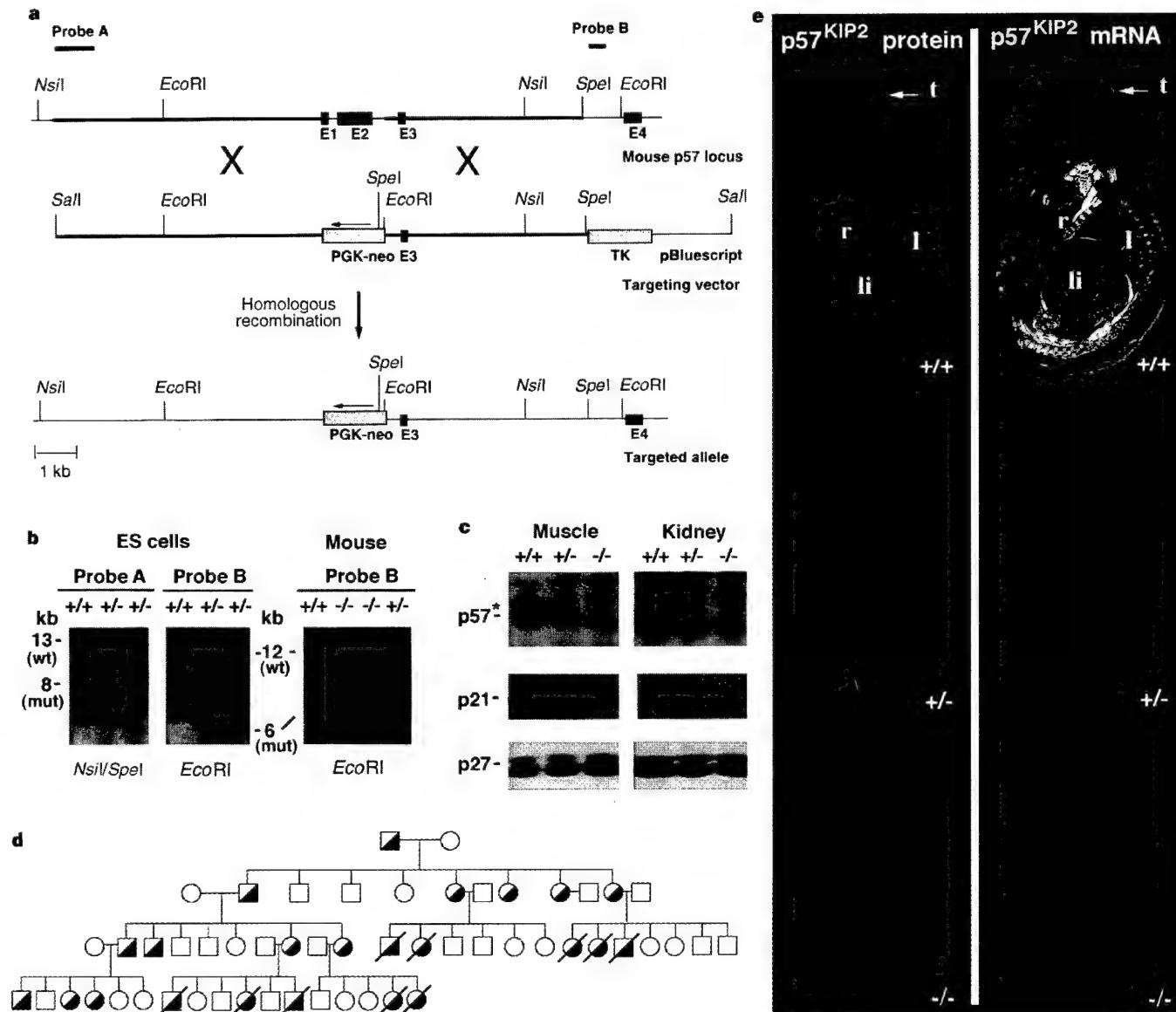


Figure 1 Targeted disruption of p57^{KIP2}. **a**, p57^{KIP2} disruption strategy. Probes (A and B) for Southern analysis are indicated. **b**, Southern blot analysis of DNA from wild-type and mutant ES clones and embryos. **c**, Western blot analysis of p57^{KIP2}, p27^{KIP1} and p21^{CIP1} proteins in muscle and kidney. The asterisk indicates a form of p57^{KIP2} resulting from phosphorylation or alternative splicing. **d**, A pedigree analysis: squares, males; circles, females. Heterozygotes are represented by

half-filled symbols. Animals displaying a mutant phenotype are indicated by diagonal lines through symbols. **e**, *In situ* hybridization and immunofluorescent analysis of sagittal sections derived from E15.5 p57^{+/+}, p57^{+/−} and p57^{−/−} embryos. A combined image of p57^{KIP2} protein (green) and nuclei stained with Hoechst dye (blue) is shown; l, lung; li, liver; r, rib; t, tectum.

null allele is lethal (Fig. 1d). The $p57^{-/-}$ animals were phenotypically indistinguishable from affected $p57^{+/-}$ heterozygotes.

Mice lacking $p57^{KIP2}$ have omphalocele

Mutant embryos showed umbilical abnormalities as early as E16.5. A herniated abdomen was noticeable in all mutants (Fig. 2A, d,e) together with malrotation of the intestines resulting in placement of the jejunum and ileum in front of the liver. Occasionally, the small intestines were found outside the abdominal cavity (omphalocele), a range of phenotypes characteristic of BWS in humans (Fig. 2A, b). Most dead or dying neonates had a slit in their abdomen where the umbilicus is normally positioned, and portions of their visceral organs were missing, presumably devoured by their mother in the process of removing the placenta and yolk sac (Fig. 2A, c).

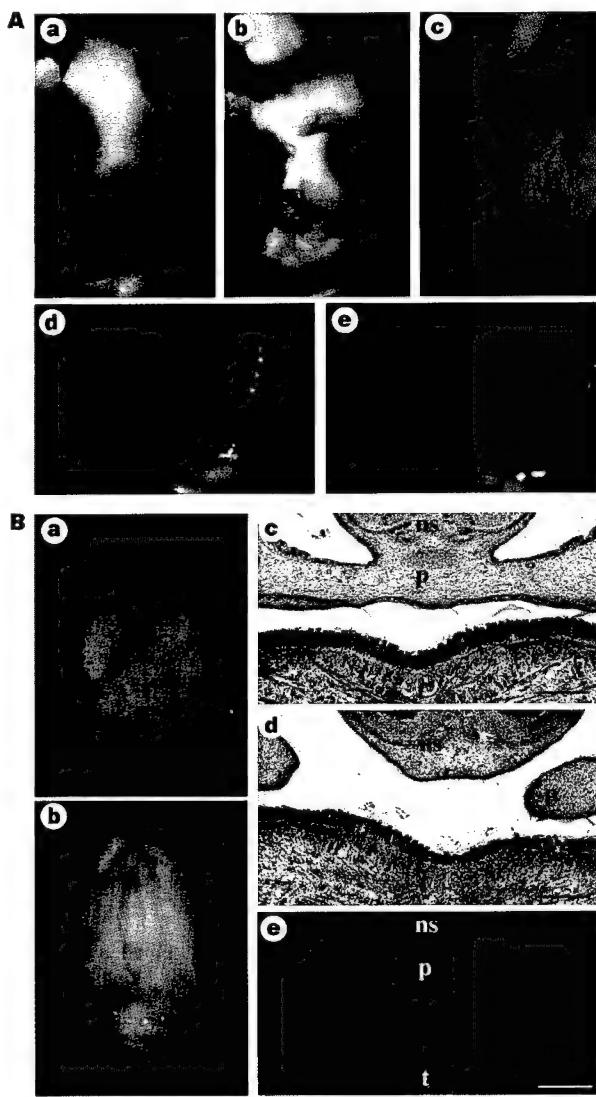


Figure 2 Omphalocele, umbilical hernia and cleft palate in $p57^{KIP2}$ mutant mice. **A.**, Intestines are found outside the abdominal cavity in some $p57^{KIP2}$ mutant newborns (**b**) but not wild-type mice (**a**). Umbilical sacs were removed in **b** to expose intestines. Typically, dead neonates (mutants) had a slit on their abdomen with missing viscera (**c**). Umbilical hernia is observed in mutants (**e**, arrow) but not in wild-type littermates (**d**). **a, b**, E20; **c**, P0; **d, e**, E18. **B.**, Jaws were removed from P0 wild-type (**a**) and mutant (**b**) mice to allow palate viewing. Haematoxylin and eosin-stained coronal sections show a fused palate in E20 wild-type (**c**) mice and cleft palate in mutant (**d**). $p57^{KIP2}$ expression (green) in the palate mesenchyme and tongue muscle of an E20 embryo (**e**); ns, nasal septum; p, palate; t, tongue. Scale bars, 200 μ m.

The small intestines develop outside the body until E15.5 (ref. 19), when then they enter the body cavity. How this occurs is unknown. It is unclear how omphalocele occurs in BWS, but the prevailing assumption is that visceral overgrowth limits abdominal space. In $p57^{KIP2}$ mutant embryos, visceral overgrowth was not evident. $p57^{KIP2}$ is highly expressed in intestinal mesenteries that form connections between the intestine folds and the abdomen and may participate in intestine re-entry. However, no obvious histological malformation was detected in mutant mesenteries.

Body wall muscle dysplasia in $p57^{KIP2}$ mutants

E18 mutants are 10% shorter than weight-matched $p57^{+/+}$ embryos (Fig. 3A, a). However, skeletons are nearly identical lengths (Fig. 3A, b), indicating a different aetiology for the body-length anomaly. Because proper musculature is required for skeletal stature, we investigated muscle organization. Histological examination of mutant embryos revealed defects in the position of body wall muscles (Fig. 3B, b,d). At the umbilicus level, the muscle did not reach as far towards the midline of the abdomen in mutants as in wild-type mice, leaving large areas of the abdominal wall uncovered by muscle. To distinguish whether this was a cause or a consequence of herniation, we examined E14.5 embryos in which a physiological omphalocele is normally present. Muscle in the mutants also failed to reach the midline (Fig. 3B, a,c), strongly suggesting that the abdominal wall defect is responsible for the subsequent umbilical herniation.

Overall muscle differentiation was not affected in $p57$ -mutant mice, as indicated by the normal muscle-fibre organization and peripheral location of nuclei. Surprisingly only 50% of muscle nuclei express $p57^{KIP2}$ (Fig. 3B, e,f). This is consistent with the hypothesis that there are two muscle lineages, MyoD and Myf5. Perhaps $p57^{KIP2}$, which is also expressed in E11.5 somites (data not shown), is expressed in only one lineage, and is required for muscle-cell migration.

Neonatal lethality in $p57^{KIP2}$ mutant mice

Cleft palate (secondary palate) and difficulty in breathing were noticeable in all mutant neonates (Fig. 2B, compare a and b). Milk was found in their lungs, and air in their stomach and intestines, causing inflation and stretching. No histological abnormalities were found in mutant diaphragm, lung, bronchi or trachea. The severity of cleft palate was variable but could compromise breathing by allowing an accumulation of liquid in the nasopharynx that is subsequently brought into the lungs by inhalation.

Closure of the secondary palate is a complex process²⁰. Vertically growing palate shelves elevate by E13 and grow together, fusing by E16 to form the secondary palate¹⁹. Analysis of E20 mutant embryos demonstrated that the palate failed to fuse but maintained normal organization (Fig. 2B, c,d). $p57^{KIP2}$ is expressed in mesenchymal cells of the palate and muscle cells of the tongue, but not nasal or oral epithelia (Fig. 2B, e). Failure of palate closure could result from defects in mesenchymal cell migration, response to induction signals, cell proliferation, or increased apoptosis caused by inappropriate proliferation. The tongue of mutant mice is not enlarged, and is therefore not interfering in palate closure.

Renal medullary dysplasia in $p57^{KIP2}$ mutants

A major histopathological finding in BWS is non-cystic medullary dysplasia and enlargement of the kidney²¹. BWS medullary pyramids are often poorly formed and exhibit abundant connective tissue stroma with widely separated renal tubules²². In $p57^{KIP2}$ mutants the size and organization of the kidney was normal (Fig. 4a-h), but the inner medullary pyramid was significantly smaller than normal. Normally at E18.5 and E20 the maturing glomeruli are juxtapamedullary and nephrons have developed long loops of Henle reaching deep into the inner medullary region (Fig. 4g). However, mutants have fewer renal tubules (loops of Henle and collecting

Table 1 Genotype frequencies of offspring* Male p57^{+/+} x female p57^{+/+}

p57 genotype	+/+	+/-
Number	32	0
Observed (%)	100	0
Expected (%)	50	50

* Male p57^{+/+} x female p57^{+/+}

p57 genotype	+/+	+/-	-/-
Number	45	37	0
Observed (%)	55	45	0
Expected (%)	25	50	25

† Male p57^{+/+} x female p57^{+/+}

p57 genotype	+/+	+/-
Number	24	27
Observed (%)	47	53
Expected (%)	50	50

† Male p57^{+/+} x female p57^{+/+}

p57 genotype	+/+	+/-	-/-
Number	16	27	14
Observed (%)	28	47	25
Expected (%)	25	50	25

* Genotyped at 2 weeks of age.

† Genotyped between E18.5 and E20.

ducts) and more stromal cells in the inner medulla (Fig. 4d,h), a phenotype very similar to that of BWS patients²¹. No defects in primary nephrogenesis were observed, indicating that ureter bud branching and mesenchymal induction occurred normally in the mutant kidney.

To determine whether medullary dysplasia is due to improper development or to regression of a properly formed kidney, a developmental analysis was performed. At E16 the renal pelvis forms through fusion of initial ureter branches. A normal renal pelvis was observed in the E16.5 mutant kidney (Fig. A, B). At this stage the inner medulla normally begins to form, but in the mutant this was totally absent. At E18.5 the mutant inner medulla has also failed to develop properly (Fig. 4C, D). This indicates that the medullary dysplasia is due to arrested or improper development.

p57^{KIP2} is expressed in podocytes of maturing glomeruli and in interstitial stromal cells between renal tubules (Fig. 4i) but not the tubules themselves. This expression pattern argues against an intrinsic defect in the renal tubule, and underscores the importance of tissue–tissue interactions between epithelia of renal tubules and surrounding mesenchymal cells during the development of the loops of Henle, as has been demonstrated previously for ureteric branching and the mesenchymal to epithelial transition.

Bone development and collagen X expression

Skeletal staining with alcian blue (cartilage) and alizarin red (bone) revealed abnormalities in mutant skeletons. At E14.5 the two sternal bands in mutants were widely separated, whereas those in wild-type animals had already fused (Fig. 5A, a,b). By E19, wild-type sternabrae were fully ossified, whereas ossification had just begun in the mutant (Fig. 5A, c,d). Two separate ossification centres in each sternbra were clearly seen in the mutant, indicating imperfect fusion of sternal bands (Fig. 5A, d). Smaller ossification centres were observed in mutants at all stages of development in forelimbs, vertebra and supraoccipital bone, as shown in Fig. 5A, e–l. Although mutant limbs are shorter, they are thicker than in the wild type (compare Fig. 5A, g and h).

Vertebrate long bones are formed through endochondral ossification, which involves formation of a cartilage framework that is converted to bone by replacement. Within this framework, cells are organized into distinct zones: the epiphyseal centre zone contains resting chondrocytes that act as stem cells for the adjacent proliferative zone, where chondrocytes proliferate and form columns, and the hypertrophic zone, containing dying chondrocytes that are in the process of ossification. Histological examination of mutant long-bone sections showed a slight disorganization of the columnar alignment of differentiating chondrocytes (Fig. 5B, a,b). Furthermore, the mutant hypertrophic zone is slightly thinner than in the wild type and contains smaller cells, suggesting impairment of chondrocyte differentiation.

p57^{KIP2} is expressed at moderate levels in resting chondrocytes, low levels in the proliferative zone, and very high levels in the hypertrophic zone (Fig. 5B, c). We examined cell-cycle withdrawal in the mutant hypertrophic zone to determine whether cell division in this zone might account for resistance to ossification. A higher level of BrdU incorporation was observed in the resting (2.2-fold) and proliferative (1.6-fold) chondrocytes of E15 mutant animals, but at later times, such as postnatal day (P)0, only a small increase in BrdU labelling was observed in resting (20%) and proliferative (14%) chondrocytes. At E18.5 there is a 10% greater cell density in these two zones, which may explain the bone thickening in mutant animals (Fig. 5A, e–h). However, no BrdU labelling was observed in either the mutant or wild-type hypertrophic zones at any stage.

Collagen X is expressed in hypertrophic chondrocytes and has been implicated in proper bone development²³. Expression of collagen X was significantly reduced in the mutant hypertrophic zone (Fig. 5B, d,e). Thus p57^{KIP2} is required for expression of collagen X, and perhaps other genes that facilitate the ossification

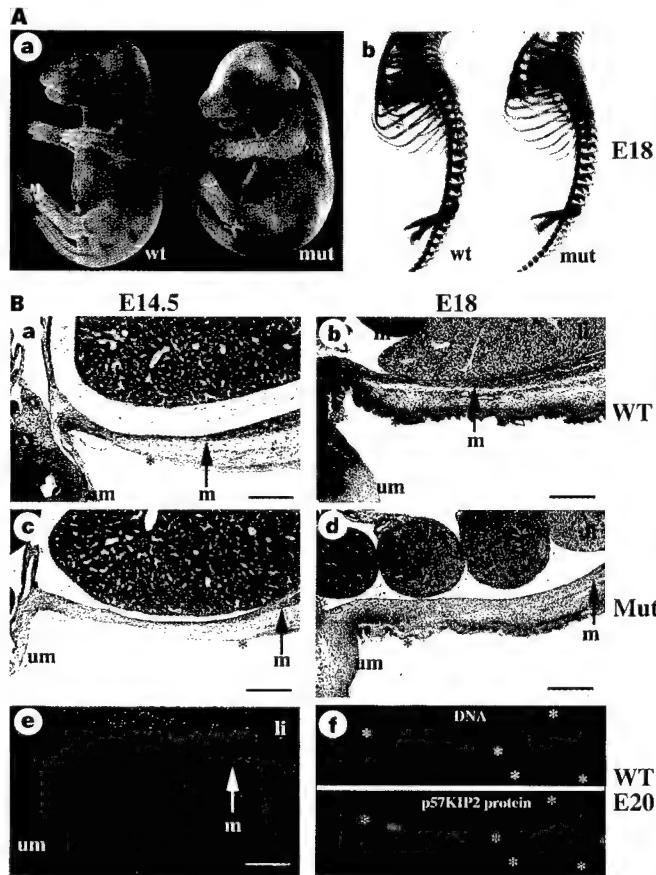


Figure 3 Body-wall dysplasia in p57^{KIP2} mutants. **A**, E18 p57^{+/+}(wt) and p57^{+/−}(mut) embryos (**a**) and skeletons (**b**) prepared from the same embryos. Mutants have shorter bodies but normal skeletal length. **B**, Transverse embryo sections were stained with haematoxylin and eosin. The distance between umbilicus and the tip of rectus abdominis muscle (arrows) is greater in mutants than in wild-type littermates in E14.5 (**a** and **c**) and E18 (**b** and **d**) embryos. Skin development is also delayed in mutants (asterisks). p57^{KIP2} protein was detected in the rectus abdominis muscle, connective tissue, skin, and liver (**e**), and is present in half of the nuclei in muscle (**f**); in, intestine; li, liver; m, muscle; um, umbilical cord. Scale bars, 200 μ m.

of chondrocytes. Because no cell-cycle entry was observed in mutant hypertrophic chondrocytes, the failure to express collagen X suggests that p57^{KIP2} may play a direct role in processes of differentiation.

Adrenal cortex hyperplasia and cytomegaly

The adrenal gland is among the most consistently enlarged organs in BWS patients and shows extensive cytomegaly. p57^{+/−m} mutants show a significant enlargement of the adrenal gland (a 25% to 100% increase in volume; Fig. 6a). Although mutant adrenals were normal histologically, there was a threefold increase in the frequency of cytomegaly (Fig. 6c,d). Expression of p57^{KIP2} is restricted to fetal adrenal cortex (Fig. 6b), indicating a role in controlling cell proliferation.

Cycling and apoptosis in lens after loss of p57^{KIP2}

The p57^{KIP2} protein is present in high levels in postmitotic lens fibre cells, with low-level, sporadic expression in the anterior epithelial layer (Fig. 7a,b). p57^{KIP2} is induced in the equatorial zone (Fig. 7a, arrows) where epithelial cells are withdrawing from the cell cycle and initiating terminal differentiation²⁴, suggesting a role for p57^{KIP2} in promoting one or both of these processes. Analysis of p57^{−/−} and p57^{+/−m} lenses revealed grossly normal lens structure at E13.5 with minor vacuolization, but a more pronounced vacuolization in E15.5 and older lenses (Fig. 7e). Late-stage differentiation markers such as γ -crystallin and membrane-intrinsic protein-26 were unaffected (data not shown). However, p57^{KIP2} loss causes inappropriate S-phase entry in lens fibre cells (Fig. 7f,g) and an increase in apoptotic nuclei (Fig. 7i,j), possibly contributing to the vacuolated appearance. Another phenotype of p57^{−/−} lenses was a 10-fold increase in apoptosis in the anterior epithelial compartment (Fig. 7j). Epithelial cells that accumulate in the centralmost position of the anterior epithelial layer are normally eliminated by a p53-independent apoptotic mechanism to maintain the single-cell layer anteriorly²⁵. Because these cells express p57^{KIP2}, the increase in apoptosis could result from an accelerated rate of proliferation and execution of a normal mechanism designed to eliminate excess cells in the central zone of the anterior lens.

Discussion

p57^{KIP2} acts as a regulator of cell proliferation in the adrenal gland, the lens epithelia and certain chondrocytes. The partial dependency on p57^{KIP2} for reducing cell proliferation reveals the redundant mechanisms used to limit tissue growth. A similar situation is observed in cell culture where agents that induce cell-cycle arrest immediately increase levels of certain CKIs and subsequently reduce the levels of the cyclins and CDKs. While undergoing the process of reducing CDK activity during differentiation, the absence of CKIs may allow additional cell cycles to occur before CDK activity is sufficiently reduced to block cell-cycle entry.

Loss of Rb is associated with increases in proliferation, impaired expression of differentiation markers, and inappropriate apoptosis in lens fibre cells²⁵. p57^{−/−} lenses show less cell proliferation and apoptosis than Rb^{−/−} lenses, thus p57^{KIP2} is likely to play a partly redundant role upstream of Rb. However, the increase in apoptosis in the anterior epithelial compartment is greater in p57^{−/−} than in Rb^{−/−} lenses. Thus, even in the same cell type, the relationship between p57^{KIP2} and Rb (and possibly other cell-cycle regulators) changes with respect to differentiation state. Furthermore, the ossification defect in p57^{−/−} mice is similar to that observed in p107^{−/−} p130^{−/−} double mutants²⁶, and it is likely that p57^{KIP2} is also regulating these proteins to control proliferation in chondrocytes.

Several tissues in p57^{−/−} mutants showed developmental defects not obviously linked directly to increased cell proliferation, such as defects in kidney development, differentiation of hypertrophic chondrocytes, muscle organization, and formation of the secondary

palate. These defects may indicate roles in differentiation distinct from the biochemical role of p57^{KIP2} as a CKI. In the case of the ossification defects, the hypertrophic chondrocytes exit the cell cycle properly but were disorganized and failed to express differentiation markers such as collagen X. Non-CDK binding regions of the p57^{KIP2} protein, such as the repeat regions or the conserved QT domains, may play cell cycle-independent roles in differentiation.

Using the p57^{KIP2}-defective mouse allows us to assess unambiguously the role of p57^{KIP2} in BWS. Its BWS-related phenotypes support a causal role for a mutation found previously¹⁷. Both patients with p57^{KIP2} mutations displayed omphalocele, macroglossia, gigantism and earlobe grooves. p57^{KIP2}-deficient mice also show omphalocele but not gigantism, macroglossia, visceromegaly and hypoglycaemia (data not shown). However, the expression pattern of p57^{KIP2} is consistent with a possible role in both gigantism

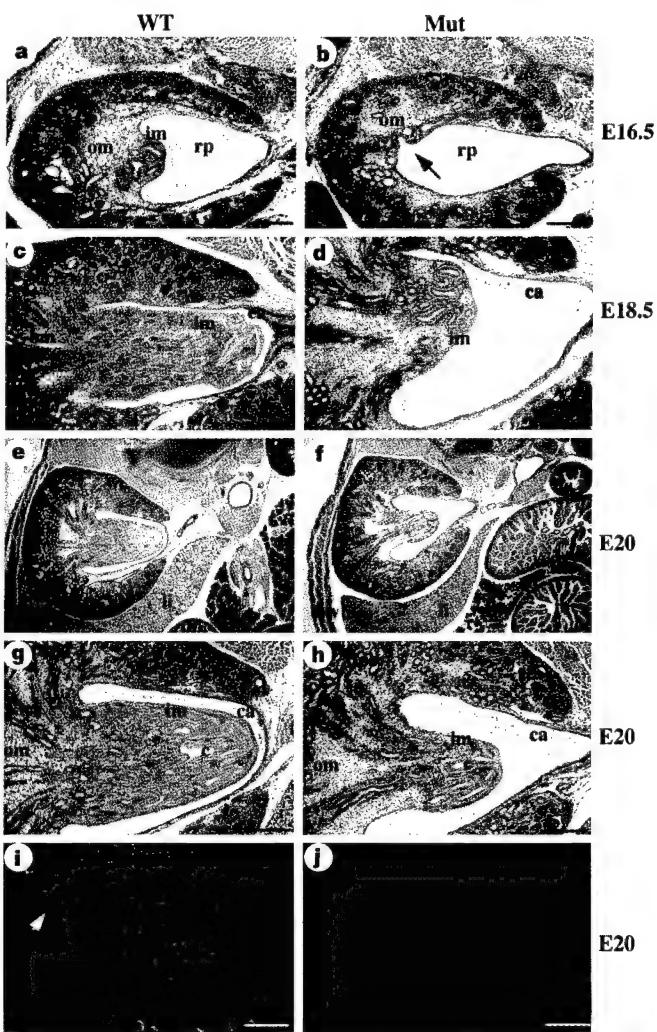


Figure 4 Kidney medullary dysplasia in p57^{KIP2} mutants. **a, b**, At E16.5, the inner medulla is absent in the p57^{−/−} mutant kidney (arrow). **c, d**, At E18.5, a delayed elongation of the inner medulla is evident in the mutant kidney. **e, f**, Low magnification shows relatively normal organization of the mutant kidney at E20. **g, h**, A higher magnification of **e** and **f**. The mutant inner medulla is significantly smaller than in the wild type, containing fewer loops of Henle (asterisks) and collecting ducts with more stromal cells in between. **i, j**, p57^{KIP2} protein is expressed in mesenchymal cells between renal tubules of the inner medulla and in podocytes of glomeruli (arrow), but not in mutants (**j**). **bw**, body wall; **c**, collecting ducts; **dpc**, distal and proximal convoluted tubules; **im**, inner medulla; **in**, intestine; **li**, liver; **om**, outer medulla; **pa**, pancreas; **ca**, renal calyx; **rc**, renal cortex; **rp**, renal pelvis. Scale bars, 200 μ m.

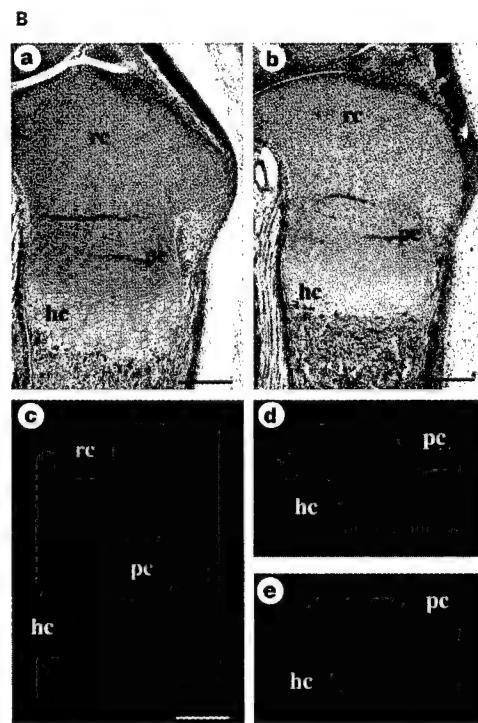
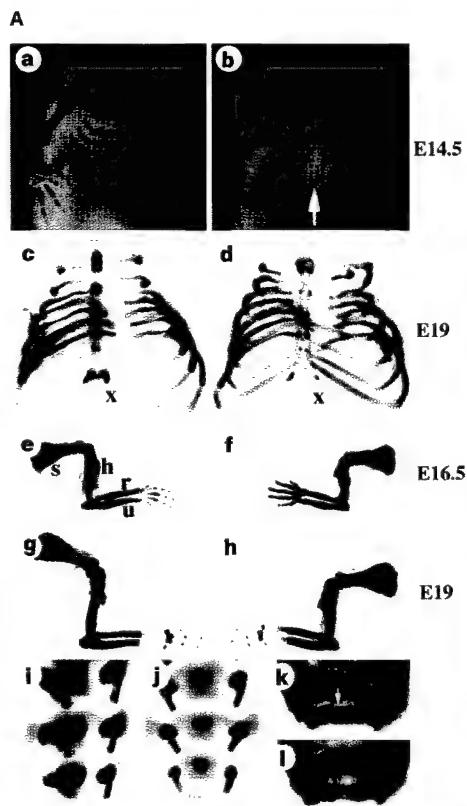


Figure 5 Impaired endochondral ossification in $p57^{KIP2}$ mutants. **A**, Alcian blue (cartilage) and alizarin red (bone) staining of E14.5 skeletons showing fused sternum rudiments in wild-type (**a**) and an unfused sternum in $p57^{H-m}$ mice (**b**, arrow). Sternebrae are fully ossified in E19 wild-type (**c**) but not mutant (**d**) embryos. Note the separate sternum ossification centres and enlargement of the xiphoid process in **d**. **e-h**, Forelimbs of E16.5 (**e**, **f**) and E19 (**g**, **h**) of wild-type (**e**, **g**) and mutant (**f**, **h**) embryos. Reduced ossification is observed in mutant E19 vertebrae (**j**) and P0 supraoccipital bone (**k**) relative to wild-type (**i**, **l**). **B**, Longitudinal sections through the tibia of E18.5 wild-type (**a**) and mutant (**b**) embryos, stained with haematoxylin and eosin. $p57^{KIP2}$ expression was visualized by immunofluorescence (**c**). Collagen X expression was detected in wild-type (**d**) but not in mutant (**e**) hypertrophic chondrocytes; **h**, humerus; **hc**, hypertrophic chondrocytes; **pc**, proliferating chondrocytes; **r**, radius; **rc**, reserve chondrocytes; **s**, scapula; **su**, supraoccipital bone; **u**, ulna; **x**, xiphoid process. Scale bars, 200 μ m.

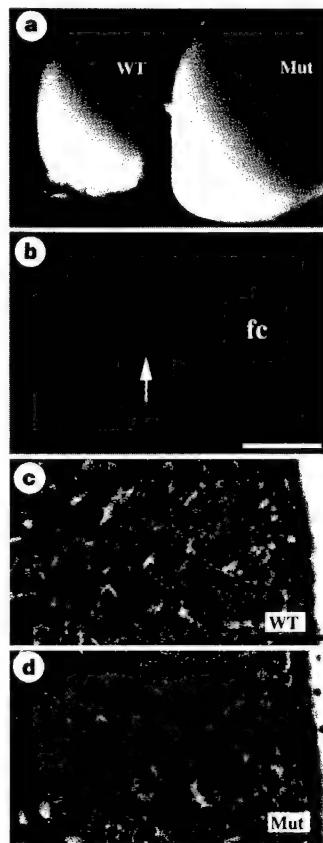


Figure 6 Hyperplasia of the adrenal gland in $p57^{KIP2}$ mutants. **a**, The E19 adrenal gland is enlarged in $p57^{H-m}$ mice. **b**, $p57^{KIP2}$ protein is expressed in the fetal cortex but not the medulla (arrow) in an E20 adrenal gland. **c, d**, Haematoxylin and eosin-stained sections of E20 wild-type (**c**) and $p57^{H-m}$ (**d**) adrenal glands. Arrows indicate cytomegaly; **fc**, fetal cortex; **m**, medulla. Scale bars: **b**, 200 μ m; **c, d**, 50 μ m.

and macroglossia; the mouse tongue has extremely high levels of $p57^{KIP2}$ during development, as does the human placenta, which controls nutrient flow to embryos^{10,11}.

It is unknown which of the internal manifestations of BWS these two patients exhibit. However, the $p57^{-/-}$ mouse displays several common internal BWS phenotypes including enlargement of the adrenal cortex, adrenal cytomegaly, and renal medullary dysplasia, which are now strongly predicted to be present in the two affected individuals. Phenotypes present in the $p57^{-/-}$ mouse not frequently considered to be associated with BWS are cleft palate, endochondral ossification defects, and ocular lens impairment. It should be noted that, unlike the mouse, human $p57^{KIP2}$ imprinting is incomplete with 5% residual expression from the paternal allele¹⁵. Therefore, although mice lacking the maternal allele are null mutants, the equivalent humans are hypomorphs. This may affect the relative penetrance of phenotypes between species. Nevertheless, increased frequency of cleft palate^{27,28}, skeletal anomalies²⁹, and cataract formation²⁹ have been reported in conjunction with BWS. A very high frequency of palate defects including cleft and soft palate have been observed in 6 of 10 BWS patients in a study from Japan²⁸. The high frequency of palate defects in that study relative to others may reflect the differences in frequencies of modifier genes in distinct populations. In response to this study it was learned that the BWS patient from a previous study¹⁸ who had a maternally inherited null allele in $p57^{KIP2}$ also had a cleft palate (I. Hatada, personal communication). This provides further compelling support for the involvement of $p57^{KIP2}$ in BWS. It has also been observed that the long bones in BWS patients often show widened metaphyses and a thickened bony cortex²¹. This is consistent with our observations of the thickened bones in $p57^{KIP2}$ -deficient mice. Thus the analysis of the $p57^{KIP2}$ -deficient mouse has not only provided proof of the role of $p57^{KIP2}$ in BWS, but has also extended the range of phenotypes expected on further analysis of BWS patients with $p57^{KIP2}$ mutations. Furthermore, it has provided an explanation for the physiological basis of several phenotypes observed in BWS patients, including skeletal abnormalities (chondrocyte proliferation and differentiation), omphalocele (abdominal

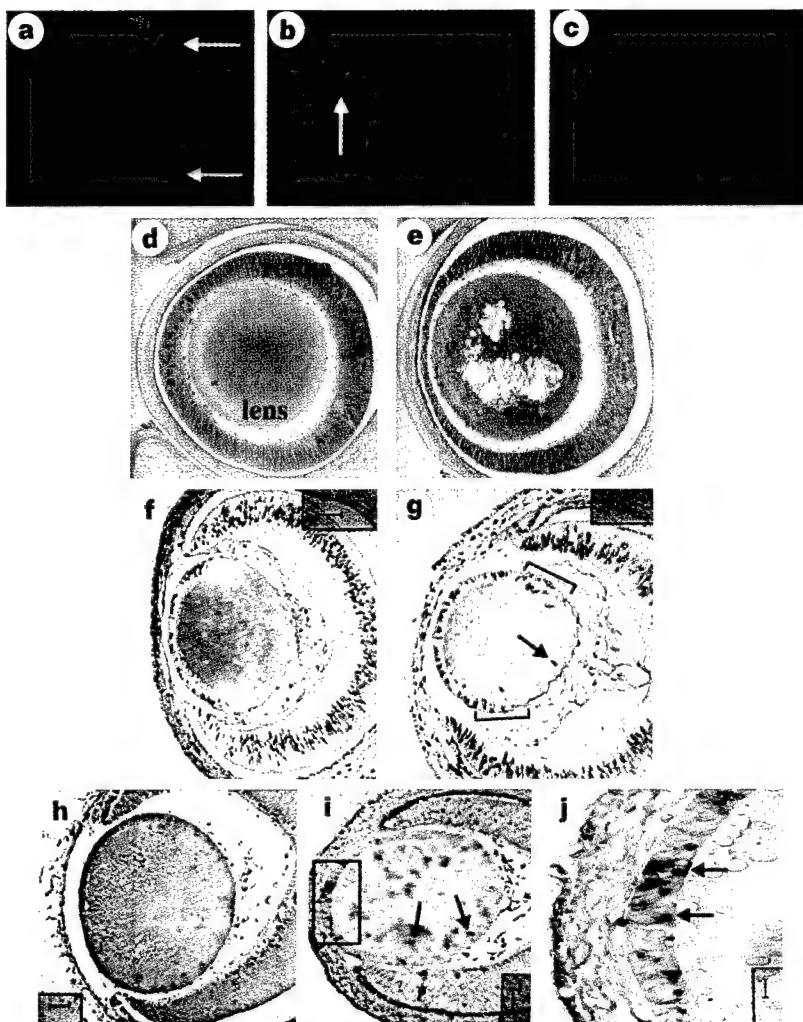


Figure 7 Loss of $p57^{KIP2}$ causes increased proliferation and apoptosis in the lens. **a-c**, Immunofluorescent analysis shows strong $p57^{KIP2}$ expression in all nuclei of the postmitotic lens fibre cell compartment (arrows) (**a**), occasional nuclear and cytoplasmic staining in anterior epithelial cells (arrow) (**b**), and no expression in $p57^{-/-}$ lenses (**c**). **d, e**, Histological presentation of haematoxylin and eosin-stained E15.5 lens sections (sagittal) derived from $p57^{+/+}$ (**d**) or $p57^{-/-}$ (**e**) embryos. **f, g**, BrdU incorporation assays on E13.5 lens sections derived from $p57^{+/+}$ (**f**) or $p57^{-/-}$ (**g**) embryos. Brackets show inappropriate S-phase entry in normally postmitotic lens fibre cells of the equatorial region. The arrow indicates S-phase entry in central lens fibre cells. **h-j**, TUNEL assays on E13.5 lens sections derived from $p57^{+/+}$ (**h**), $p57^{-/-}$ (**i**), $p57^{-/-}$ (**j**) embryos (higher magnification of the boxed region in **i** showing abundant apoptotic nuclei in the anterior epithelial layer). Scale bars: **f-i**, $10\ \mu\text{m}$; **j**, $4\ \mu\text{m}$.

muscle developmental defects) and adrenomegaly (absence of a cell proliferation inhibitor).

Because $p57^{KIP2}$ is central to BWS, it is likely that most or all BWS forms affect $p57^{KIP2}$ function in some way. Loss of $p57^{KIP2}$ could account for BWS involving alterations at the maternal 11p15.5 locus. However, it is more difficult to imagine how $p57^{KIP2}$ is directly involved in the form of BWS caused by duplication of the paternal 11p15.5 region. It is possible that duplicating the imprinted paternal $p57^{KIP2}$ allele could affect the maternal allele's expression, but this seems unlikely. An alternative explanation is the linked-antagonist model, which proposes that mutations in $p57^{KIP2}$ or overexpression of a $p57^{KIP2}$ antagonist cause BWS. The chromosome specificity of the paternal duplication could be accounted for by exclusive paternal expression of the antagonist, or by the fact that, owing to tight linkage, the duplicated region would also carry $p57^{KIP2}$, and so duplications including the maternal $p57^{KIP2}$ would not produce BWS. Under these conditions, duplication of the paternal 11p15 region would have an effect similar to mutating the $p57^{KIP2}$ gene, increasing a dosage-dependent antagonist. It is possible that IGF2 is the linked antagonist that acts in the same pathway as $p57^{KIP2}$, but in opposition. Increased IGF2 expression leads to some phenotypes associated with BWS in mice³⁰ and humans³¹. The linked-antagonist model predicts that each mutation (or increased dosage) would have some common effects (for example, omphalocele, macroglossia and gigantism) and some unique effects (for example, earlobe creases, viceromegaly and renal medullary dysplasia), and there would be significant variability in the range of different phenotypes, depending on which genes were mutant or over-

produced. Paternal uniparental disomy would cause the most severe phenotype because all $p57^{KIP2}$ expression would be lost and increased dosage of the antagonist(s) would occur. The linked-antagonist model accounts for the highly variable penetrance associated with this syndrome.

Because we now have an understanding of the molecular defects that cause BWS, there should be a concerted effort to correlate phenotype with genotype of BWS patients to establish whether groups of phenotypes are linked and correlate with a particular genotype. This analysis should help establish whether BWS is a single disease or a collection of similar diseases that operate through an overlapping pathway, and will definitively establish whether $p57^{KIP2}$ is a tumour suppressor. □

Methods

Targeting vector construction. The $p57^{KIP2}$ targeting vector was constructed using plasmids containing *PGK-neo* and *pMC1-HSV-tk*. *PGK-neo* on a *Xba*I-*Hind*III fragment was cloned into $p57\text{Sal}1\text{d}3$ cleaved with *Hind*III and partly with *Xba*I to make $p57\text{KO-2A}$, containing a 7-kb fragment of $p57^{KIP2}$ 5'-genomic DNA. A 5.6-kb *Spe*I-*Eco*RV fragment of $p57^{KIP2}$ 3'-genomic DNA was ligated into *Xba*I-*Cl*I (blunt)-digested *pMC1TK* generating $p57\text{KO-2B}$ with *tk* gene 3' to $p57^{KIP2}$ genomic DNA. To generate the targeting vector $p57\text{KO-2}$, the *Sal*I-*Eco*RV fragment of $p57\text{KO-2A}$ containing $p57^{KIP2}$ 5'-genomic DNA and *neo* was ligated into *Sal*I-*Cl*I (blunt)-digested $p57\text{KO-2B}$. $p57\text{KO-2}$ introduces *Eco*RI and *Spe*I sites into $p57^{KIP2}$ locus.

ES cell culture and germline transmission of targeted allele. Linearized $p57\text{KO-2}$ ($25\ \mu\text{g}$) was electroporated into 1.1×10^7 AB2.1 ES cells and cultured³². Homologous recombinant clones were microinjected into 3.5-d.p.c.

C57BL/6 blastocysts and a male chimaera derived from clone p57-2.2.12B transmitted the targeted allele.

Gross and histological analysis. Embryos were fixed in 10% formalin or Bouin's. For histological analysis, fixed samples were dehydrated using ascending concentrations of ethanol, cleared in xylene and embedded in paraffin wax. Embedded samples were sectioned at 5 μ m.

Immunohistochemistry and *in situ* hybridization. Immunostaining for collagen X was performed as described²³. For cell proliferation assays, pregnant mice were injected with BrdU (0.1 mg per g body weight) 2 h before caesarean section, and positive cells were identified with an anti-BrdU monoclonal antibody (Dako). *In situ* hybridization was performed as described⁵. Apoptosis and BrdU incorporation assays were performed as described²⁵.

Protein analysis. Tissues from E18 embryos were lysed in NP-40 buffer and cleared by centrifugation. Protein (30 μ g) was immunoblotted with anti-p57^{KIP2} (monoclonal antibody KP90)³³, anti-p21^{CIP1} (monoclonal antibody 65)⁶ or anti-p27^{KIP1} antibodies (provided by A. Koff) using ECL detection.

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**p53-Independent Expression of p21^{Cip1} in
Muscle and Other Terminally Differentiating Cells**

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Terminal differentiation is coupled to withdrawal from the cell cycle. The cyclin-dependent kinase inhibitor (CKI) p21^{Cip1} is transcriptionally regulated by p53 and can induce growth arrest. CKIs are therefore potential mediators of developmental control of cell proliferation. The expression pattern of mouse p21 correlated with terminal differentiation of multiple cell lineages including skeletal muscle, cartilage, skin, and nasal epithelium in a p53-independent manner. Although the muscle-specific transcription factor MyoD is sufficient to activate p21 expression in 10T1/2 cells, p21 was expressed in myogenic cells of mice lacking the genes encoding MyoD and myogenin, demonstrating that p21 expression does not require these transcription factors. The p21 protein may function during development as an inducible growth inhibitor that contributes to cell cycle exit and differentiation.

Proper development of a multicellular organism is complex and requires precise spatial and temporal control of cell proliferation. A large network of regulatory genes has evolved to specify when and where in the embryo cells divide. This control is superimposed upon the basic cell cycle regulatory machinery.

Cell proliferation requires the action of cyclins, which serve as activators of their cognate cyclin-dependent kinases (Cdks). (1) D- and E-type cyclins have been implicated in controlling passage through the "re-

striction" point (2), after which cells become committed to a round of cell division (3). G₁ cyclin accumulation is required for cell cycle entry and members of this family, particularly D-cyclins, have been identified as targets of growth factors (1).

Equally important in the execution of developmental programs is the arrest of growth once the program is complete. Whereas the control of terminal differentiation may be mediated by multiple, possibly redundant, mechanisms, cell cycle arrest through inactivation of Cdks is likely to be a central feature. Possible mediators of such negative control are two classes of Cdk inhibitory (CKI) proteins typified by p21^{Cip1} (4) and p16^{INK4/MTS1} (5). The p21 protein inhibits G₁ cyclin complexes containing Cdk2, Cdk3, Cdk4, and Cdk6 and is transcriptionally induced by overexpression of the tumor suppressor protein p53 (6) or by activation of p53 after DNA damage, consistent with a role for p21 in the p53-dependent G₁ checkpoint (7).

The ability of p21 to function as an inducible cell cycle inhibitor suggests that it

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might also function to mediate cell cycle arrest during development. Thus, knowledge of the timing and location of p21 expression in the embryo could provide evidence that this CKI participates in terminal differentiation in a developing organism. Because p53 regulates transcription of p21 in vitro (6, 7), we also tested whether p21 expression in vivo was dependent upon p53.

We used *in situ* hybridization (ISH) to probe for p21 expression during mouse embryogenesis (8). Embryos of day 7.5 post coitum (p.c.) (0 to 5 somites) showed no expression of p21. By day 8.5 p.c., we detected hybridization along the midline of the neural tube and in the hindgut. Presomitic paraxial mesoderm did not express p21, but there was hybridization in the dermamyotome (Fig. 1A), where the first determined

myocytes are localized. By day 10 p.c., there was strong expression of p21 in the muscle fibers extending from the anterior to the posterior margin of the myotome (Fig. 1B). Hybridization of adjacent sections with a probe for the muscle-specific basic-loop-helix protein myogenin (9) exhibited signals in the same regions in which p21 was detected (Fig. 1, C and G). Muscle cells in the myotome are post-mitotic (10). A transverse section through a day-10 p.c. embryo at the position of the forelimb revealed expression of p21 in the developing limb (Fig. 1D). In the section shown, there is a zone of hybridization in the dorsal region of the limb mesenchyme, representing the emerging dorsal muscle mass. Muscle primordia in the limb expressed the homeobox gene Pax3 (11) in the same region where p21 was expressed

(Fig. 1, D and E). Later in development, limb and intercostal muscle strongly expressed p21 (Fig. 1, H through J).

At days 13 to 15 p.c. we detected expression of p21 in nasal epithelium, tongue muscles, hair follicles, the outermost layer of embryonic epidermis, and cartilage (Fig. 1, H through M). With the exception of cartilage, which has been studied in less detail, each of these sites of expression contain post-mitotic, differentiated cells (10). This is also true of the apical ectodermal ridge (AER) where expression of p21 was observed as early as day 10 p.c. (Fig. 1F). During limb outgrowth, the AER maintains the underlying mesenchymal tissue in an undifferentiated state, but the cells of the AER itself do not divide. Selective expression of p21 in differentiated epithelium was evident in the nasal region. At day 15.5 p.c., p21 was expressed throughout the post-mitotic respiratory epithelium, whereas the adjacent olfactory epithelium had a delimited pattern of expression (Fig. 1N). We detected no p21 mRNA in the mitotic germinal layer of the olfactory epithelium, but p21 was expressed in the layer containing differentiating olfactory neurons (Fig. 1N).

The mRNA encoding p53 is widely expressed in the day-12 p.c. embryo (Fig. 2A) (12), suggesting that normal amounts of p53 are not sufficient for p21 induction in many cell types. To determine whether expression of p21 was dependent upon p53, we examined expression of p21 in sections of mice lacking the gene encoding p53 [p53^(-/-)] (13). Mice that lack p53 develop normally but incur tumors much more rapidly than do wild-type animals (13, 14). As judged by ISH, expression of p21 during early embryogenesis was independent of expression of p53 (Fig. 2A). Sagittal sections through day-11.5 p.c. embryos lacking p53 stained strongly for p21 in somites (Fig. 2A). A survey of the major sites of p21 expression, including cartilage, nasal epithelium, intercostal tongue, and limb muscle from day-12 to 14.5 p.c. embryos lacking p53 revealed that p21 expression in these tissues was independent of p53 expression (Fig. 2A).

We determined expression of p21 in adult mouse tissues (Fig. 2C). Because adult tissues are a primary target for tumorigenesis, it was conceivable that p21 expression in certain adult tissues is p53-dependent. Analysis of small intestine and stomach revealed that p21 is expressed in a highly selective manner and is found in large amounts only in the fully differentiated columnar epithelium (Fig. 2B). The p21 mRNA was absent from the embryonic brain and spinal cord, but large amounts of p21 mRNA were detected in the adult brain (Fig. 2C), especially in the olfactory bulbs (Fig. 2B). Uniform expression of p21 was observed in adult lungs, heart, and skeletal muscle (Fig. 2B). In all of

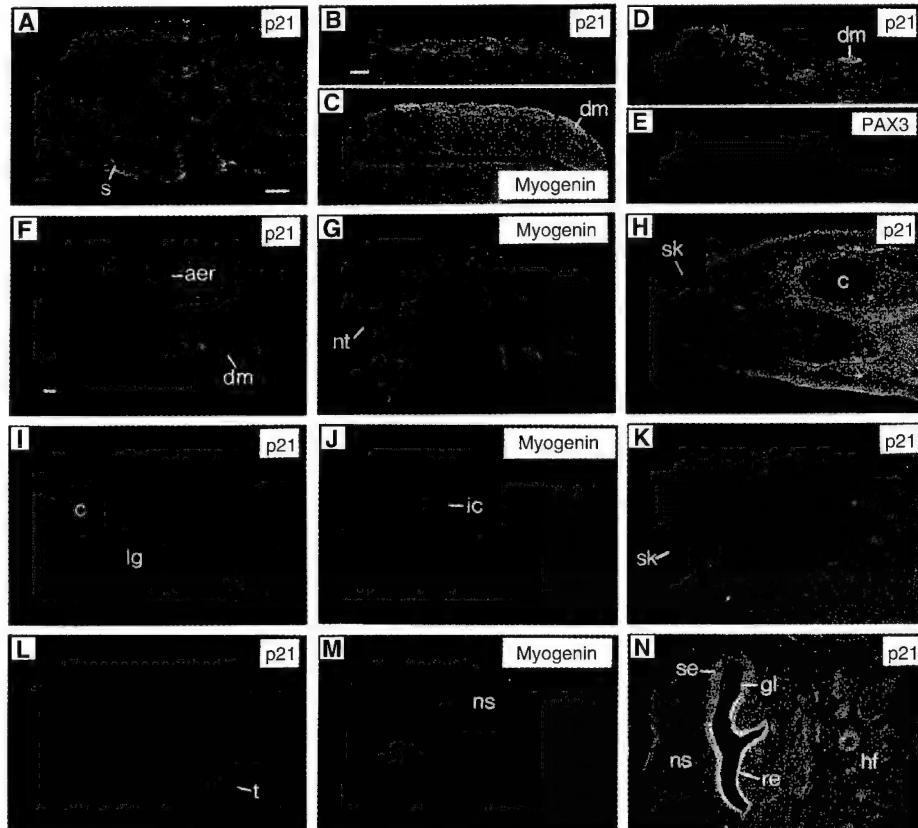
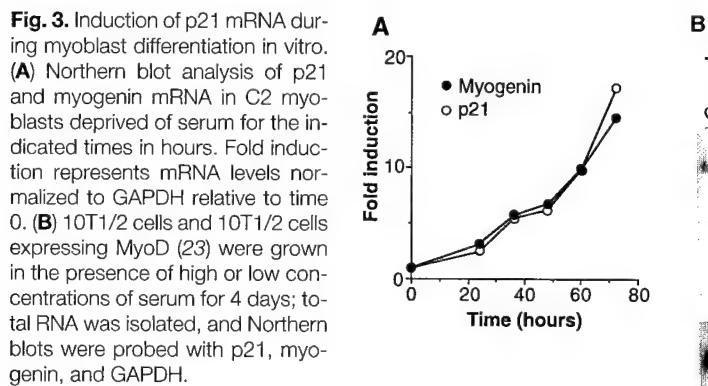
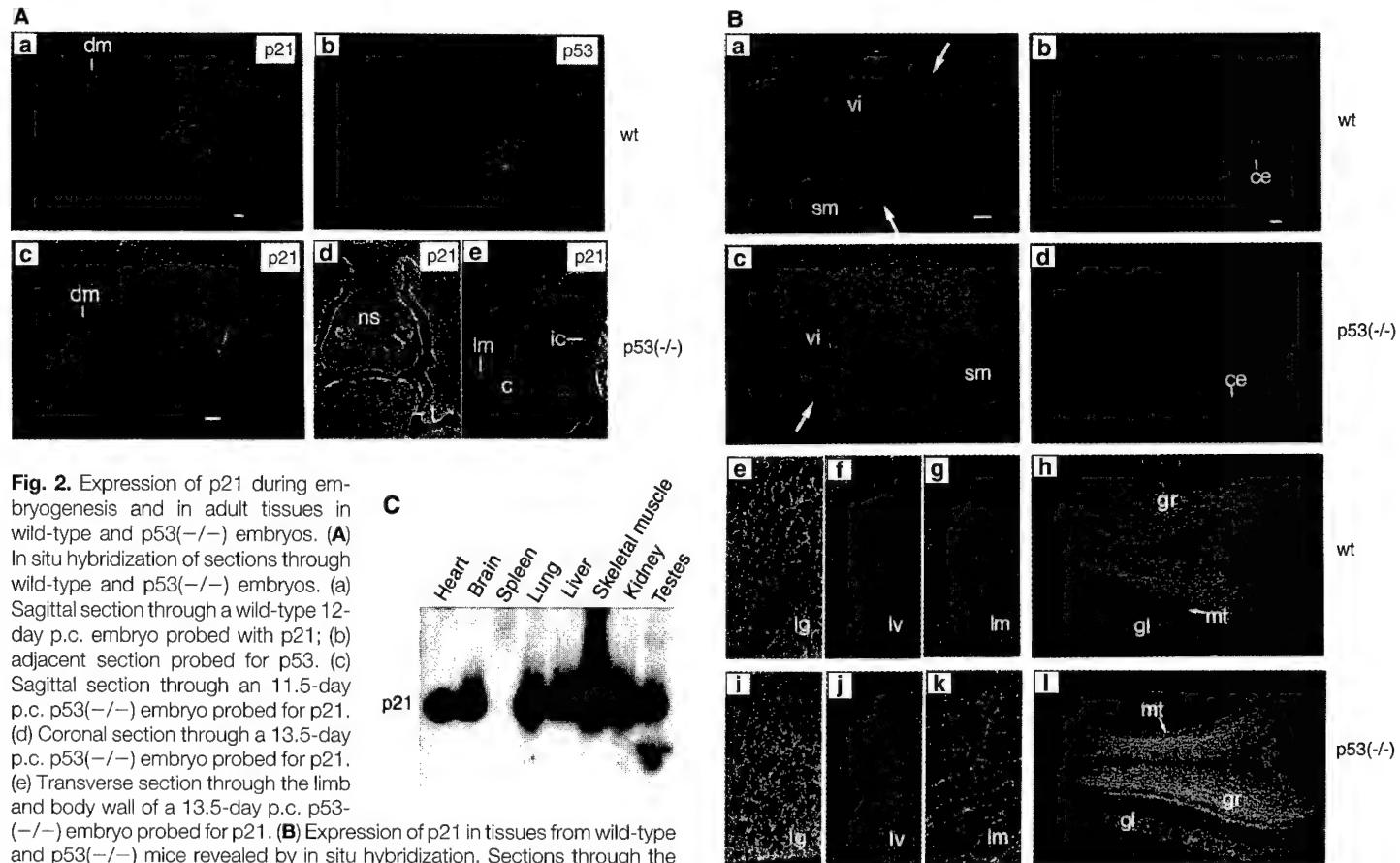
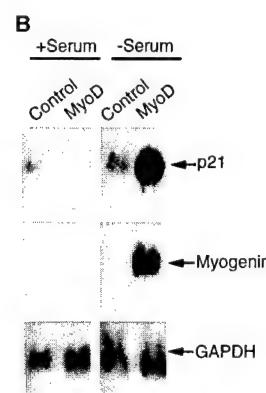


Fig. 1. Expression of p21, myogenin, and Pax3 during mouse embryogenesis. Sections are from C57 black embryos and were subjected to *in situ* hybridization with the indicated ³⁵S-labeled riboprobes (8). (A) Transverse section through an 8.5-day p.c. embryo: p21 probe. (B and C) Adjacent transverse sections through a 10-day p.c. embryo: p21 probe (B), myogenin probe (C). (D and E) Adjacent transverse sections through the limb bud and the body wall of a 10-day p.c. embryo: p21 probe (D), Pax3 probe (E). (F and G) Adjacent transverse section through a 10-day p.c. embryo: p21 probe (F), myogenin probe (G). (H and K) Cross-section through a 15.5-day p.c. forelimb: p21 probe. (K) Higher magnification of H. (I and J) Nearby transverse sections through the body wall and spinal cord of a 12.5-day p.c. embryo: p21 probe (I), myogenin probe (J). (L and M) Adjacent coronal sections illustrating p21 expression (L) in the nasal cavity and tongue muscles of a 12.5-day p.c. embryo, (M) was hybridized with a myogenin probe. (N) Coronal section through the nasal cavity of a 15.5-day p.c. embryo: p21 probe. Abbreviations: aer, apical ectodermal ridge; c, cartilage; dm, dermamyotome; gl, supporting cells; hf, hair follicle; lg, lung; ic, intercostal muscle; ns, nasal septum; nt, neural tube; re, respiratory epithelium; s, somite; se, olfactory sensory epithelium; sk, skin; and t, tongue. A, D, E, and K are the same magnification; scale bar in A is 50 μ m. B, C, H, I, J, and N are the same magnification; scale bar in B is 100 μ m. F, G, L, and M are the same magnification; scale bar in F is 100 μ m. Hybridization signal is in red.



the adult tissues analyzed, p21 expression was unaltered in p53(-/-) mice (Fig. 2B). Consistent with this, mRNA prepared from a typical tissue, the stomach, showed no p53-dependent changes in the amount of p21 mRNA (15).

Our expression studies suggest that p21 may function in muscle cell differentiation. The myogenic program is controlled by helix-loop-helix transcription factors of the



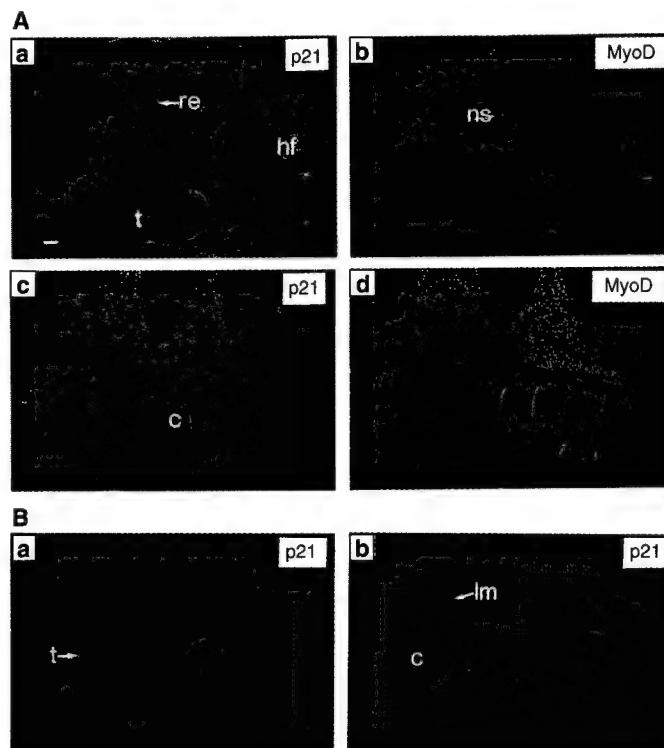
MyoD family and expression of either MyoD, Myf5, or myogenin is sufficient to convert a number of cell types into muscle (9, 16). Whereas Myf5 and MyoD can functionally replace each other in vivo (17), myogenin has a separate function and is required for the formation of differentiated muscle fibers (18). A critical step in this differentiation process is cell cycle arrest, and overexpression of MyoD in various cell types leads to a

and applies to A(a,b). Scale bar in A and (c) is 100 μ m and applies to c through e. Scale bar in B(a) is 100 μ m and applies to c, e through g, and i through k. Scale bar in B(b) is 100 μ m and applies to d, h, and l. **(C)** Expression of p21 mRNA in adult mouse tissues by Northern analysis. Tissue blots of polyadenylated RNA (Clontech) were probed with 32 P-labeled mouse p21 cDNA.

block in DNA replication (20). The process of muscle cell differentiation can be mimicked in vitro in C2 myoblasts, which form post-mitotic, multinucleated myotubes when grown in the absence of growth factors (19). Consistent with a role for p21 in the differentiation process, withdrawal of serum from C2 myoblasts results in induction of p21 mRNA as detected by Northern analysis (Fig. 3) (21). After 72 hours, at which time \sim 50% of the cells have been incorporated into myotubes, p21 mRNA levels had increased by 17-fold. The time course for myogenin mRNA induction was similar to that of p21 mRNA. Furthermore, MyoD expression in 10T1/2 cells in low serum was sufficient to induce both p21 (Fig. 3B) (22) and differentiation into myotubes (23).

To examine whether p21 expression is dependent upon myogenin, ISH analysis was done with mice lacking the gene encoding myogenin [myogenin(-/-)]. These animals produce muscle precursor cells expressing MyoD and Myf5 but lack fully differentiated muscle fibers and die shortly after birth (18). Expression of p21 was retained in myoblasts

Fig. 4. Expression of p21 in myogenin(-/-) embryos and embryos lacking both MyoD and myogenin. **A**(a, b) Adjacent coronal sections, illustrating nasal cavity, tongue muscle, and hair follicles of 15.5-day p.c. myogenin(-/-) embryo sections, were probed with p21 (a) or MyoD (b) to identify muscle precursor cells. (c and d) Cross-sections through the forelimb of a 15.5-day p.c. myogenin(-/-) embryo probed with p21 (c) or MyoD (d). **B**(a, b) Sections through the tongue (a) and forelimb (b) of a 14-day p.c. MyoD(-/-); myogenin(-/-) embryo probed with p21. Abbreviations: c, cartilage; hf, hair follicle; lm, limb muscle; ns, nasal septum; re, respiratory epithelium; and t, tongue. Scale bar in A(a) is 100 μ m and applies to all panels in Fig. 4.



in the forelimb and tongue of myogenin(-/-) embryos and in other muscle tissue (Fig. 4A), indicating that myogenin is not required for p21 induction during myogenesis. Although there is a block to myoblast differentiation in myogenin(-/-) mice, BrdU (bromodeoxyuridine)-labeling experiments indicate that the undifferentiated myoblasts that populate the presumptive muscle-forming regions withdraw from the cell cycle normally (24).

Although MyoD expression is sufficient to induce p21 (Fig. 3B) (22), p21 is expressed in somites at day 8.5 p.c. (Fig. 1A) before expression of MyoD at day 10.5 p.c. (9), consistent with the possibility that other transcription factors may control p21 induction during muscle cell differentiation in the embryo. One caveat is that at day 8.5, MyoD may be expressed in amounts sufficient to activate p21 but below the limit of detection by ISH. However, at day 14 p.c., p21 was expressed in muscle precursor cells from forelimb and tongue in MyoD(-/-); myogenin(-/-) mice in amounts comparable to that found in wild-type animals (Fig. 4B).

Our results revealed a strong correlation between arrest of cell proliferation and p21 expression in vivo. This correlation was particularly evident in the skeletal muscle lineage where p21 expression was similar to that of myogenin in vivo and in vitro. Although MyoD is sufficient to arrest the cell cycle and induce muscle differentiation, neither MyoD nor myogenin is required for p21 regulation in vivo, suggesting a possibly redundant role for Myf5. Other CKIs and neg-

ative cell cycle regulators, such as the retinoblastoma gene product Rb (28), may also contribute to differentiation of muscle and other cell lineages. Although the basal amount of p21 in fibroblast cell lines in vitro is p53-dependent (26), p21 expression in the embryo and adult does not require p53. Taken together, our results indicate that p21 functions as an inducible growth inhibitor both during development and in G₁ checkpoint control, and that p53's role in p21 expression is likely to be limited to the checkpoint function.

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Cdk inhibitors: on the threshold of checkpoints and development

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The regulation of cyclin-dependent kinases is at the heart of cell cycle control and, by inference, the control of cell proliferation. Recent advances in regulation of these kinases have uncovered a group of small proteins that bind to and inhibit them, thus preventing cell cycle progression. Linking these proteins to tumor suppressor functions has provided a much sought after connection between cancer and cell cycle control.

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Introduction

Cell proliferation is controlled via an intricate network of extracellular and intracellular signaling pathways which process both negative and positive growth signals. The ultimate recipients of many of these signals are cyclin-dependent kinases (Cdks), a family of enzymes which catalyze events required for individual cell-cycle transitions. Cdks require association with cyclins for activation, and the timing of Cdk activation is dependent largely upon the timing of cyclin expression (reviewed in [1-3]). In mammalian cells, positive growth signals ultimately lead to expression of G₁ cyclins, which serve as essential activators of Cdks 2, 3, 4 and 6, facilitating passage through the restriction point (reviewed in [4-6]). Cells transiting this point are generally committed to the completion of a full cell cycle, even in the absence of growth factors. In unicellular eukaryotes such as budding and fission yeast, a single protein kinase (Cdc28 or cdc2⁺, respectively; cdc stands for cell-division-cycle) fulfills the function of the family of proteins found in multicellular organisms [2]. Presumably, the larger number of Cdks in higher eukaryotes reflects the increased regulatory capacity required to execute the complex instructions of development.

Coordination of the timing and order of cell cycle events is critical for high-fidelity transmission of genetic information. Consequently, a number of biochemical pathways, called checkpoints (reviewed in [7-9]), have evolved to ensure that the initiation of particular cell cycle events is dependent upon the successful completion of others. Checkpoints also monitor the physical integrity of chromosomes and coordinate cell-cycle transitions. Cdk activities are potential targets of checkpoint function.

The regulation of Cdks has been a subject of intense investigation [1,3]. Many of the molecular mechanisms of

regulation have been elucidated in outline form; however, it is still not clear which if any of these mechanisms are utilized by particular checkpoints to control cycle transitions. This lack of clarity results partially from the linear dependency relationships within the cell cycle: if a stimulus blocks a particular event, it is not clear whether that is because it arrests the cell cycle at a point preceding the execution of that event, or whether there is a direct regulatory connection. Until recently, our knowledge of the mechanisms of Cdk regulation consisted of positive regulation by cyclin abundance, phosphorylation by Cdk-activating kinase (CAK) and dephosphorylation by the Cdc25 phosphatase, and negative regulation by inhibitory tyrosine phosphorylation through the Wee1 and Mik1 proteins [1,3]. Recently, a new class of negative regulators has emerged: the Cdk inhibitors.

The discovery of genes encoding Cdk inhibitors (Table 1) has had a major impact on our understanding of the fundamental mechanisms underlying negative growth control and checkpoint function [10-12]. The universality of negative cell cycle control via Cdk inhibitors is indicated by the identification of inhibitors in budding and fission yeast, as well as in mammalian cells. The fact that cells contain multiple types of Cdk inhibitors, some of which function to inhibit the same kinases, suggests that these proteins are involved in independent regulatory networks that have common targets for effecting cell-cycle arrest. Loss of negative growth control and an inability to appropriately monitor the fidelity of DNA replication are common features of tumor cells. Elucidation of mechanisms which disrupt Cdk inhibitor function should therefore contribute to our understanding of the process of cellular transformation. It is also likely that negative growth factor pathways that function to arrest the cell cycle during development also utilize Cdk inhibitors. This review summarizes current

Abbreviations

CAK—Cdk-activating kinase; Cdk—cyclin-dependent kinase; PCNA—proliferating cell nuclear antigen; pol⁸—DNA polymerase 8; RF—replication factor; TGF—transforming growth factor.

knowledge in the rapidly moving field of Cdk inhibitors in both mammalian and yeast systems.

Table 1. Cdk inhibitors.

Inhibitor	Species	Primary Cdk target	Comments
Far1	<i>S. cerevisiae</i>	Cln-Cdc28	Blocks the cell cycle in G ₁ in response to α -factor
Sic1	<i>S. cerevisiae</i>	Clb5,6-Cdc28	Regulates entry into S phase
rum1	<i>S. pombe</i>	Cdc13-Cdc2	Regulates entry into S phase
p16	Human, mouse	Cdk4, Cdk6	Candidate tumor-suppressor gene
p21	Human, mouse	A-, D- and E-cyclin complexes	Transcriptionally activated by p53 after DNA damage leading to G ₁ arrest
p27	Human, mouse	D- and E-cyclin complexes	Inhibits Cdk2 and Cdk4 complexes related to p21

Cdk inhibitors in yeast: extrinsic and intrinsic control of cell-cycle transitions

SIC1: an intrinsic regulator of the cell cycle

The first Cdk-inhibitory factor was detected in *Saccharomyces cerevisiae* as a protein found associated with Cdc28 [13,14•]. The gene encoding this protein was cloned and called both *SIC1*[15••] and *SDB25* [16]. Sic1 was found to play a non-essential role in the G₁/S phase transition in a normal cell cycle. Its abundance is tightly cell-cycle regulated by transcription and proteolysis, and it binds to and inhibits the cyclin complexes Clb5-Cdc28 and Clb6-Cdc28. Following passage through Start, Sic1 is destroyed, thereby activating the Clb5 and Clb6 kinases, which are necessary for efficient S phase activity [17••]. Proteolytic destruction of Sic1 is dependent upon *CDC34*, which encodes an E2 ubiquitin conjugating enzyme. Temperature-sensitive *cdc34* mutants arrest with a 1C DNA content at the non-permissive temperature, whereas *cdc34sic1* double mutants arrest with replicated DNA. Thus, Sic1 is a negative regulator of DNA synthesis.

Far1 links negative growth factors to cell cycle arrest

FAR1 was identified as a mutant that failed to arrest the cell cycle of *MATa* cells in response to α -factor, but that maintained integrity of the transcriptional response to α -factor [18]. It was subsequently shown to bind to and inhibit Cdc28. *FAR1* is regulated both transcriptionally and post-transcriptionally: its mRNA is induced by α -factor, and it is phosphorylated by the α -

factor responsive *FUS3* kinase [19,20••], a modification required for Far1 activity. As Far1 can inhibit Cdc28, which is required for several cell-cycle transitions, how does it gain specificity for arresting cells in G₁? Apparently Far1 shows some specificity for Cln1-Cdc28 and Cln2-Cdc28 complexes (which are active in G₁) whereas it is unable to inhibit Clb2 or Clb5 kinase complexes [21••]. A second possibility which remains to be explored is that the stability of Far1 is cell cycle regulated *in vivo* [22].

Too much *rum1* and S phase gets out of control

The *rum1* gene was identified in screen for cDNAs from *Schizosaccharomyces pombe* that are lethal when expressed at high levels [23••]. It has the remarkable property when overproduced of allowing multiple rounds of DNA synthesis in the absence of mitosis. The ability of *S. pombe* *cdc2* mutants to re-establish the S phase state after heat shock had been established earlier [24] and it was suggested that Rum1 might act to block the mitotic form of Cdc2. It has since been shown to inhibit Cdc2 kinase activity *in vitro* (J Correa, S Moreno, P Nurse, personal communication). *rum1* is not essential and *rum1* mutants fail to arrest in G₁ in response to nutrient limitation or *cdc10* inactivation, arresting with a G₂ DNA content. (*cdc10* is a temperature-sensitive mutation that arrests *S. pombe* at the restrictive temperature in G₁ prior to DNA replication.) This is consistent with a role as an inhibitor of Start, which is somewhat at odds with its overproduction phenotype. Perhaps it is both an inhibitor of Start and mitosis, and a stabilizer of the S phase state. This might be possible by Rum1 binding to and inhibiting the G₁ and M forms of Cdc2, while binding to and failing to inhibit S phase forms, thereby preventing other inhibitors from functioning. It remains to be determined how and if Rum1 is regulated in response to starvation.

Mammalian inhibitors: the cell cycle meets cancer

p21

p21 is currently the best studied Cdk inhibitor. This is due largely to the fact that it was discovered in several laboratories using completely different approaches that simultaneously identified p21 as a Cdk inhibitor (Cip1, p21, p20CAP1 [25••,26••,27]), as a gene regulated by the tumor suppressor protein p53 (Waf1 [28••]) and as a protein that is likely to play a role in the inability of senescent cells to enter the cell cycle (Sdi1 [29••]). In normal fibroblasts, p21 is associated with a variety of Cdk-cyclin complexes, including Cdk2 associated with cyclins A and E, and Cdk4 associated with D-type cyclins [25••,26••,30•,31••], and is also found weakly associated with Cdc2-cyclin B [31••]. *In vitro*, p21 is a potent inhibitor of Cdk2 and Cdk4 (Ki <10 nM) and is a much poorer inhibitor of Cdc2. p21 associates preferentially with the Cdk-cyclin complex and is found in either cyclin or Cdk immune complexes. This is in con-

trast with p16 which appears to disrupt the Cdk4-cyclin D complex via association with Cdk4 (see below).

The identification of *p21* as a p53-regulated gene [28^{**}, 32^{**}] has provided significant insight into understanding how this critical tumor suppressor protein functions. p53 is a transcription factor that is involved in at least three functions: cell cycle arrest in G₁ in response to DNA damage; transcriptional induction of DNA damage inducible genes; and regulation of apoptosis in response to γ -irradiation [33,34]. Through a sensory pathway that has not been elucidated, p53 levels increase in response to DNA damage, correlating with increased transcription of p53-regulated genes. The promoter of the *p21* gene contains two consensus p53-binding sites [28^{**}]. The available data are consistent with a model in which p21 is a primary mediator of the p53-dependent G₁ arrest pathway. Treatment of cells containing wild-type p53 (but not mutant p53) with DNA-damaging agents leads to induction of p21 protein and accumulation of p21 in Cdk2-cyclin E immune complexes, resulting in reduced kinase activity [32^{**},35]. G₁ arrest is presumably due to the reduced activity of Cdk2-cyclin E and perhaps of other Cdks whose activities are required for the G₁/S transition. It is likely that p53 regulates other important genes in addition to p21 during G₁ arrest; however, it is noteworthy that overexpression of p21 alone in normal fibroblasts is sufficient for cell cycle arrest [25^{**},29^{**}]. Many transformed tissue culture cell lines containing inactive p53 also lack p21 in Cdk immune complexes [28^{**},31^{**},32^{**}]. Thus it is possible that the basal level of p21 expression in these cells is at least partially dependent upon p53 function. Given a potential role for p21 in other checkpoint functions and development, however, it is likely that this gene is regulated by pathways that are independent of p53 [36]. The fact that p53 is one of the most commonly mutated genes in human cancer [37-39] points to the potential importance of p21 in negative growth control. That mutants in p21 have yet to be identified in human cancers suggests that p21 may not be the only p53-regulated gene involved in growth control.

Normal diploid fibroblasts differ from transformed cells in that many Cdk-cyclin complexes contain proliferating cell nuclear antigen (PCNA) in addition to p21 [30^{*}]. PCNA plays a positive role in cell proliferation in that it is required for processive DNA replication carried out by DNA polymerase δ (pol δ). This polymerase catalyzes leading and lagging strand synthesis and depends on the activity of replication factor (RF) C, with which it associates. PCNA, which exists as a trimer, associates with the RFC/pol δ complex and 'clamps' it to the DNA template, thereby enhancing processivity. It has recently been shown *in vitro* that p21 has the ability to block pol δ -dependent SV40 DNA replication through its association with PCNA in crude cell extracts requiring SV40 T-antigen, as well as in a purified system utilizing only PCNA, RFC and pol δ [40^{**},41^{**}]. This inhibition can be overcome by addition of excess PCNA but not RFC or pol δ , consistent with the idea that p21 interacts directly with PCNA. Interestingly, p21 efficiently

inhibits the elongation step of polymerization, probably by disturbing processivity, but does not efficiently disrupt the initiation function of PCNA [41^{**}]. Presumably this function of p21 slows DNA replication in S phase to allow repair processes time to correct damage, thus preventing replication of damaged templates. Although not yet verified *in vivo*, these data suggest that p21 plays a dual role in the DNA-damage checkpoint by both arresting the cell cycle through inhibition of Cdks and blocking DNA replication. As PCNA also functions in excision repair, additional studies are required to test whether p21 can also block this function of PCNA.

p27

Some cells, such as mink lung fibroblasts, arrest the cell cycle in G₁ in response to extracellular signals such as transforming growth factor (TGF) β . Early studies found that one of the outcomes of TGF- β treatment or of contact inhibition was the accumulation of an inactive form of Cdk2-cyclin E containing a 27 kDa inhibitor, p27KIP1 [42,43]. The cDNA for p27 was cloned independently using protein sequence information [44^{**}] and the two-hybrid system with Cdk4-cyclin D as the target [45^{**}]. The amino-terminal 60 residues of p27 are similar in sequence (44% identity) to the amino-terminal portion of p21. *In vitro*, p27 fusion proteins inhibit the same spectrum of Cdks as p21, although additional studies using purified components are needed to assess the relative potencies of the two inhibitors towards the various kinase complexes. Association of p27 with newly formed Cdk2-cyclin E complexes can also block phosphorylation of Cdk2 on Thr160 by the activating enzyme CAK [44^{**}], providing a second mode of regulation. How is p27 regulated by TGF- β and other growth arrest pathways? p27 is not transcriptionally regulated by TGF- β in the cell types that have been examined [44^{**}]; instead, accumulation of p27 in Cdk2-cyclin E complexes appears to be triggered by altering the availability (or perhaps the activity) of p27 [44^{**},46]. TGF- β has been shown to repress Cdk4 synthesis leading to a reduction in Cdk4-cyclin D complexes [47] that harbor the majority of p27 in cycling cells [45^{**}] and potentially freeing p27 to inhibit other Cdks. Alterations in the balance of active Cdk-cyclin complexes through redistribution of inhibitors may be a common theme in cellular growth control.

p16

In normal cells, Cdk4 is associated with a D-type cyclin, PCNA and p21 [30^{*}]. In some transformed cells, however, Cdk4 is found associated with p16 and these complexes lack a cyclin and PCNA [31^{**}]. D-type cyclins in these cells lack an associated kinase subunit. p16 proved to be a potent inhibitor of Cdk4-cyclin D kinases [48^{**}]. On the basis of the Cdk4-p16 association in transformed cells, it is thought that binding of p16 to Cdk4 blocks and/or disrupts cyclin association [48^{**}]; however, this idea has not been verified *in vitro* using isolated components. Unlike p21 and p27, p16 appears to be a selective

inhibitor of kinases that are activated by D-type cyclins (Cdk4 and Cdk6), suggesting that it may play a more specialized role in cell cycle control. The indication that p16 has structural homologs (MTS2 [49•]) suggests that there is much more to be learned about this class of Cdk inhibitor.

p16 has not yet been placed in a regulatory pathway. The finding that it is frequently inactivated in tumor-derived cell lines, typically through deletion [49•,50•], indicated that it plays an important role in negative growth control. Indeed, the p16 gene was isolated independently during positional cloning of genes in a region of human chromosome 9p21 that is frequently deleted in melanomas and gliomas [49•,50•]. As many as 70% of tumor cell lines from various tumor types contain alterations at the p16 (MTS1) locus. It is altered at a lower, but significant, frequency in primary tumors (10 to 20%; [49•,50•,51,52]). That an inhibitor of cyclin D kinases acts as a tumor suppressor is consistent with the previous discovery of cyclin D1 as the *PRAD1* oncogene and its amplification in many tumors [4]. Thus activation of cyclin D kinase activity by increasing kinase concentration (cyclin D overexpression) or removing inhibitory factors (mutations in p16, reduction of p21) may represent one of the central mechanisms resulting in loss of growth control in cancer.

Conclusions

The multitude of Cdk inhibitors has given us pause to rethink the nature of the control of cell-cycle transitions. The results from the preliminary characterization of the yeast inhibitors have demonstrated that order and timing of cell-cycle transitions can be facilitated by inhibitors. These inhibitors may provide additional layers of regulation to reinforce control of particular transitions, as has been suggested for *SIC1*. They may also serve unique functions in controlling cell cycle arrest in response to negative growth stimuli, such as the role of *num1* in nutrient starvation and of *FAR1* in the α -factor response. Finally, it is clear from the little that has already been discovered about the regulation of these inhibitors that all forms of transcriptional and post-transcriptional regulatory mechanisms will be employed to control their levels and activity. Homologs of these inhibitors will undoubtedly be found in higher eukaryotes.

Studies of developmental regulatory pathways in higher eukaryotes have focused on growth stimulatory signals and signal transduction pathways during early embryonic development, a period of rapid cell proliferation. Equally important in the complete execution of developmental instructions is the arrest of growth once the program is completed. It is likely that many overlapping and redundant mechanisms will come into play to ensure that the cells of certain tissues and structures permanently exit the cell cycle. The high-level expression of Cdk inhibitors would be a perfect mechanism to carry out such a function. The pattern of expression of

these inhibitors during development should prove to be particularly informative in this regard.

These inhibitors could also serve among the eventual targets of morphogenic signaling pathways, providing a threshold that growth stimulatory signals must overcome to direct binary decisions such as cell growth versus differentiation. By acting as regulable buffers, these inhibitors are versatile messengers of growth arrest signals that can act together to integrate a variety of signaling pathways, contributing to the combinatorial complexity required in the development and morphogenesis of complex multicellular organisms.

It is clear that we have barely scratched the surface of the biology of inhibitor function. It is likely that many more inhibitors will be discovered with roles in growth control and cell cycle checkpoints, perhaps targeting specific kinases. Likewise, the regulation of these inhibitors promises to be complex, judging from what has already been observed. More mechanisms that negatively regulate the inhibitors are also likely to appear. For example, transient cell cycle arrest after DNA damage is apparently followed by 'inactivation' of accumulated p21, possibly via proteolytic destruction or phosphorylation. These and other mechanisms will provide much needed insight into the complex control of cell-cycle transitions and cell proliferation.

Note added in proof

A p16 homolog, p15^{INK4B}/MTS2, has been identified recently as a Cdk4/Cdk6 inhibitor [53]. Unlike p27, p15 appears to be transcriptionally regulated by TGF- β . In addition, recent studies have provided further evidence of a role for loss of p16 in cancer [54–56].

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Cdk inhibitors in development and cancer

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Activation of cyclin-dependent kinases is the primary control point of cell proliferation. Recent advances in the understanding of cell-cycle regulation have uncovered two families of proteins that bind and inhibit these kinases. These proteins are likely mediators of development and differentiation, and may provide molecular connections between the pathways of development and tumorigenesis.

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Abbreviations

Cdk	cyclin-dependent kinase
CKI	Cdk inhibitor
INK	inhibitor of Cdk
TGF	transforming growth factor

Introduction

Proper development of a multicellular organism requires precise spatial and temporal control of cell proliferation. A large network of regulatory genes has evolved to specify when and where in the organism cells divide. This network is superimposed onto the basic cell-cycle regulatory machinery, at the center of which lies the cyclin-dependent kinases (Cdks). These kinases are positively regulated by cyclin association and are required for cell-cycle progression (reviewed in [1,2]).

While much is known about how cells enter the cell cycle, relatively little is known about the strategies involved in exit from the cell cycle and maintenance of the non-proliferative state. This state is of critical importance because the vast majority of animal cells exist in a non-proliferative state throughout adult life. The inability to halt growth appropriately can lead to malformation during organism development, and to cancer. Thus, equally important to the correct execution of developmental programs is the arrest of growth once the program is complete. Additional control circuits, called checkpoints, operate during cell proliferation to maintain the order and timing of cell-cycle events. Checkpoints also monitor the integrity of DNA and mediate cell-cycle arrest and repair processes in response to DNA damage (reviewed in [3]).

Given the central role of Cdks in cell-cycle progression, it was anticipated that mechanisms responsible

for cell-cycle arrest would include alterations in Cdk activity. Conversely, mechanisms leading to increased Cdk activity would be expected to promote uncontrolled cell proliferation. The discovery of multiple classes of Cdk inhibitors (CKIs) has provided new paradigms for understanding how cell proliferation is modulated in response to a variety of extracellular and intracellular signals. Moreover, the finding that some CKIs act as tumor suppressors has provided a direct link between tumorigenesis and the loss of negative control pathways directly affecting Cdk activity. The identification of CKIs in single-cell eukaryotes (reviewed in [4,5]) in addition to mammals points to the universality of this mechanism for cell-cycle arrest.

The field of CKI activity and cell proliferation is moving rapidly; more than 130 related papers have emerged since the discovery of CKIs in late 1993, accompanied by reviews summarizing various aspects of the field [1,2,4-6]. In this review, we focus on the relationships between CKIs, developmental control of cell proliferation, and tumorigenesis.

Cyclin-dependent kinase inhibitors (CKIs)

Mammals possess two classes of CKIs (Table 1) which differ in structure, mechanism of inhibition, and specificity. One class, the p21 family, comprises p21CIP1/WAF1, the product of a p53-regulated gene and the first mammalian CKI to be discovered [7-10], and two other CKIs, p27KIP1 [11,12] and p57KIP2 [13[•],14[•]]. These inhibitors have a conserved amino-terminal 60-residue domain responsible for kinase binding and inhibition [15[•],16,17], and preferentially inhibit Cdks of the G₁/S phase [7,8,12,13[•]-15[•],16]. Interestingly, a portion of the Cdk-binding domain is found in an otherwise unrelated negative regulator, p107, and probably mediates its interaction with the Cdk complex [18[•]]. p21 is a dual specificity inhibitor in that it not only binds to Cdks but also associates with the DNA replication factor PCNA [12,19] via the former's unique carboxy-terminal domain [17,20[•]]. This interaction blocks DNA replication catalyzed by the pol-δ/RFC/PCNA complex *in vitro* [19,21], and overexpression of this domain in mammalian cells has been reported to reduce the fraction of cells found in the S phase cells [16]. p27 and p57 contain closely related carboxy-terminal sequences, called the QT domain [13[•]], which are distinct from that found in p21 and are likely to mediate interactions with other proteins.

An unusual feature of the p21 family is that multiple inhibitor molecules are required for Cdk inhibition, such that complexes containing a single inhibitor molecule are active *in vitro* (J Roberts and R Sheaff, personal communication) [15[•],22[•]]. Such active forms of a CKI

Table 1

Mammalian cyclin-dependent kinase inhibitors (CKIs).

Inhibitor	Primary target	Chromosomal location	Regulators	Comments
Ankyrin family				
p15INK4b	Cdk4,-6	9p21	TGF β [25 **]	Frequently deleted in glioblastoma [26]
p16INK4a	Cdk4,-6	9p21	?	Tumor suppressor (melanoma) [30 ** ,31 **]
p18INK4c	Cdk4,-6	1p32	?	Not yet associated with cancer
p19INK4d	Cdk4,-6	19p13	?	Not yet associated with cancer
Dual-specificity family				
p21CIP1/WAF1	Cdk2,-3,-4,-6	6p21	p53 [9],TGF β [60] MyoD [46 ** ,47 *]	Functions in G ₁ checkpoint [43 **]
p27KIP1	Cdk2,-4,-6	12p12-13	rapamycin [61] cAMP [62]	Possible tumor suppressor in prostate [57]
p57KIP2	Cdk2,-3,-4	11p15.5	?	Not yet associated with cancer [58,59] Candidate tumor suppressor for both sporadic and familial cancers

complex containing p21 and p57 can be readily found in extracts from cycling tissue-culture cells (JW Harper and SJ Elledge, unpublished data) [15 ** ,22 **]. These multidomain CKIs potentially function as adapters, which recruit substrates into active kinase complexes via their carboxy-terminal domain. Thus, under certain conditions, these proteins might actively participate in Cdk signalling pathways. Although little is known about how the levels of these proteins are regulated, it has been shown that p27, but not p21, is destroyed via a ubiquitin-proteosome pathway [23 **].

The second class of CKIs, referred to as the INK4 (Inhibitor of Cdk4) family, is comprised of ankyrin repeat proteins and includes p15, p16, p18 and p19 (Table 1) [24,25 ** ,26,27 ** –29 **]. p16 [30 ** ,31 **] and, to a lesser extent, p15 [26] are found mutated or deleted in certain types of human cancers (reviewed in [32]), and several of these p16 mutations have been shown to be non-functional in cell-cycle arrest and Cdk4 inhibition [33 ** ,34 **]. In contrast to the p21 family, INK4 family members are selective for complexes of cyclin D with Cdk4 or Cdk6, and are not found associated with active kinase complexes. INK4 homologs can associate tightly with either the monomeric Cdk subunit or the cyclin-bound form [28 **], unlike the dual-specificity class, for which association with Cdks is largely cyclin-dependent [15 **]. Although INK4 homologs cannot directly displace cyclin D from pre-formed Cdk complexes *in vitro* [28 **], these CKIs can form complexes with Cdks at the expense of cyclin D in cells [24,27 ** ,28 **]. This suggests that p16 homologs may block some aspect of Cdk/cyclin D assembly *in vivo*.

An important consequence of the existence of multiple classes of CKIs with differing specificities is that stimulus-directed increases in the levels of a particular inhibitor can cause redistribution of complex species within the existing pool of Cdk-CKI complexes [35,36]. The clearest example comes from mink lung epithelial cells where treatment with transforming growth factor- β (TGF- β) leads to induction of p15 and displacement of p27

from existing cyclin D-Cdk4-p27 complexes [36]. These alterations in subunit distribution may be required for cell-cycle arrest, although this remains to be demonstrated rigorously.

p21 participates in the p53-dependent G₁ DNA damage checkpoint

In response to DNA damage, mammalian cells arrest the cell cycle via a process that is dependent upon the tumor-suppressor protein p53 (reviewed in [3,37]). Although the number of points at which the cycle can be arrested have not been fully elucidated, it is clear that entry into S phase is blocked by arrest in G₁. In addition, p53 participates in a G₂ checkpoint that controls the ploidy of cells [38 **]. Not all cell types respond to DNA damage by arrest in G₁. For example, thymocytes undergo p53-dependent apoptosis in response to ionizing radiation [39,40]. The manner in which cell-cycle arrest and apoptosis are coordinated is only poorly understood.

The discovery that p21 is transcriptionally regulated by p53 provided an important link between the function of the major human tumor suppressor and negative cell-cycle control [7,10,41,42]. Although this relationship provided an attractive rationale for p53's tumor-suppression function, it now appears that p53's role in tumor suppression is more complex. The contribution of p21 to p53 function has recently been tested directly through the creation of mice lacking p21 [43 **].

This work has revealed that p21 is involved in only a subset of p53-dependent functions. p21 $^{-/-}$ fibroblasts are significantly deficient in their ability to arrest in G₁ in response to DNA damage or nucleotide pool perturbation, but some G₁-arrest potential exists in these mutants, pointing to a second p53-dependent G₁-arrest pathway. Yet both the G₂ spindle checkpoint and thymocytic apoptosis induced by DNA damage are normal (Fig. 1). In stark contrast to mice that lack p53, p21-deficient mice do not have increased rates of spontaneous tumors and appear to develop normally at the current level of analysis [43 **].

Although a more in-depth analysis of p21-deficient mice is warranted, and subtle phenotypes may eventually emerge, these results suggest that the role of p53 as a tumor suppressor is complex and cannot be explained solely by its ability to induce cell-cycle arrest through p21. It is possible that p53's apoptotic function or a combination of apoptotic and G₁ checkpoint functions are central to p53's tumor-suppressor function. These findings also provide a ready explanation for the rarity of mutations in the p21 gene in human tumors [44]. Significant questions left unanswered include the nature of second G₁ checkpoint pathway (Fig. 1) and the effects of DNA damage on tumorigenesis in p21-deficient mice. The second G₁ checkpoint pathway may involve inhibition of Cdk4 through tyrosine phosphorylation, as has been recently suggested for the ultraviolet (UV) irradiation G₁ checkpoint [45**].

Potential roles for CKIs in development

The successful collaboration between proliferation and morphogenesis requires the permanent cessation of cell-cycle entry once a particular structure is achieved. Thus, multiple redundant pathways are likely to operate to keep cells out of cycle once these pathways have been executed and terminal differentiation has ensued. The ability of CKIs to arrest cell proliferation when present at high levels has led to the suggestion that these versatile molecules may contribute to either initial cell-cycle exit during differentiation or to the maintenance of the arrested state.

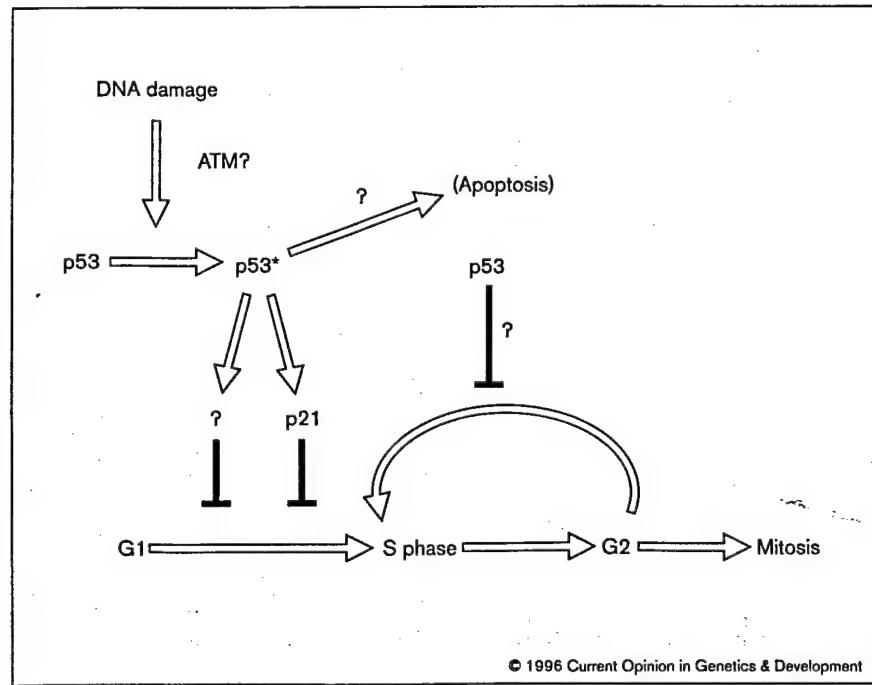
Evidence of a role for CKIs in such processes has come from an analysis of the expression patterns of dual-speci-

ficity CKIs during mouse embryogenesis [13**,46**], in adult tissues [13**,46**,47*,48*], and through an analysis of *in vitro* cell-differentiation systems [46**,47*,49,50,51**,52,53]. Both p21 and p57 are expressed in multiple cell lineages during embryonic development [13**,46**]. Importantly, the sites of expression frequently correlate with terminally arrested cells, suggesting a role for these inhibitors in maintaining cells in a non-proliferative state. Early in development, p21 and p57 are expressed at high levels in cells that have committed to the muscle differentiation pathway [13**,46**]. Later in development, p21 appears to be utilized in cells of epithelial origin, including epithelium of the stomach, intestine, prostate, and nasal organs, in addition to skin and specialized neural organs such as the olfaction bulb [44,46**]. In contrast, p57 is found at highest levels in the brain, smooth muscle of the stomach, and the lens of the eye [13**]. p21 and p57 expression overlap in several adult tissues including liver, muscle, lung and, to some extent, kidney.

The highly selective expression of p21 and p57 during development points to the complex regulatory pathways that control CKI utilization, and suggests that CKIs have evolved to perform specialized functions. It should also be noted that although these molecules may play a role in terminal differentiation, they might also help to control the many starts and stops in proliferation that cells must undergo during execution of the developmental plan. Although expression patterns of other CKIs during development have not yet been examined, it is likely

Figure 1

A current view of the integrated actions of p21 and p53. DNA damage leads to stabilization and activation of p53 by poorly defined mechanisms. The activated p53 protein (p53*) induces transcription of p21 and other genes involved in cell-cycle arrest and DNA repair. Under certain conditions, which are not completely defined, p53 activation can lead to apoptosis. In response to γ -irradiation, p53-dependent cell-cycle arrest in G₁ phase functions by both p21-dependent and p21-independent mechanisms which are only partially redundant. The second pathway may involve tyrosine phosphorylation of Cdk4, as suggested recently [45**]. p53-dependent apoptosis in response to γ -irradiation does not require p21. p53 has also been shown to regulate re-entry into S phase when mitosis is blocked by anti-microtubule agents [38**]; this function is independent of p21. p53 may also regulate ploidy in the absence of spindle interference because untreated p53^{-/-} cells rapidly increase ploidy with increased passage.



that at least some will be utilized in the aforementioned capacities.

How might developmental circuits that integrate cell-cycle exit and terminal differentiation be connected? Examination of one of the best understood differentiation system, myogenesis, has provided a clue. Expression and activation of MyoD, a basic HLH transcription factor, is sufficient to induce cell-cycle arrest and activate the muscle-cell differentiation program in various cell types. Evidence for a circuit linking CKIs, MyoD and cyclins comes from the finding that p21 expression is activated by MyoD [46^{**},51^{**}], correlating with terminal differentiation, and that forced expression of cyclin D1 in myoblasts under differentiation-inducing conditions can maintain MyoD in its non-functional form, thereby blocking differentiation [54^{**},55^{**}].

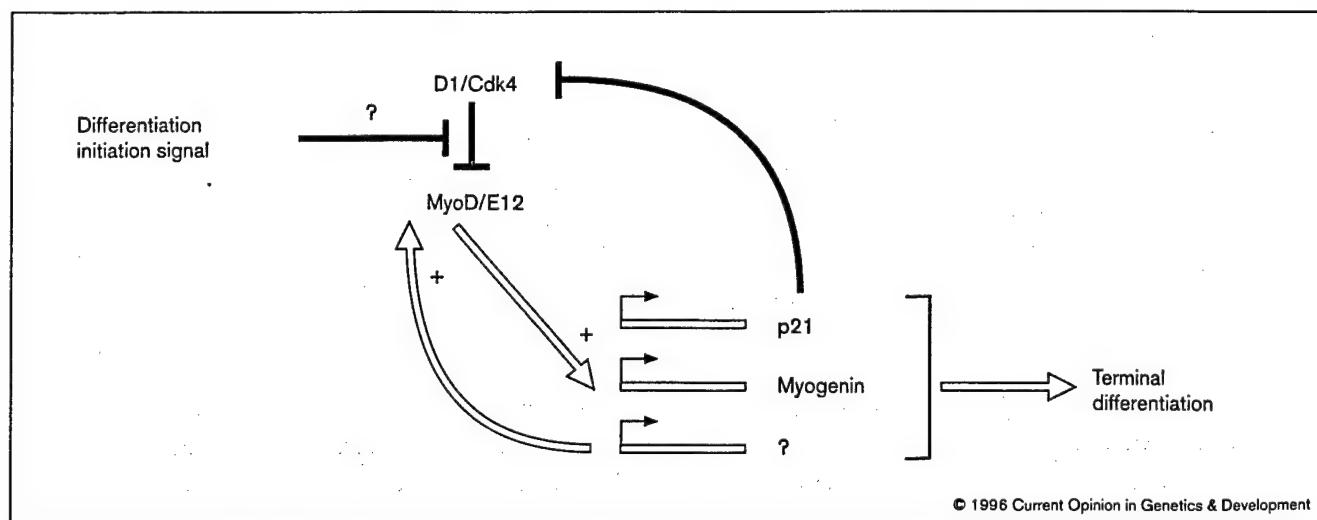
These observations suggest a differentiation switch model (Fig. 2) whereby active Cdks maintain differentiation factors such as MyoD in an inactive form in cycling cells. Once the differentiation initiation signal is received, differentiation factors are activated and induce the expression of cell-cycle inhibitors that block Cdks, both potentiating differentiation factor activity and locking cells in G₁. It is not known whether the initial signal functions by inhibiting Cdks or by circumventing their inhibitory function. Additional genes may then be induced to maintain cells in the non-proliferative state.

The finding that p21 is induced during myoblast differentiation implicated this CKI as an important mediator of this process. It is now clear, however, that activation of p21 expression by MyoD is not required for muscle-cell development, as mice that lack p21 still develop muscle [43^{**}]. It is likely that p21 is redundant with other negative regulators, such as Rb, in this tissue. Previous studies have shown that Rb plays a key role in maintaining differentiated myotubes out of cycle. Consistent with this, we have found that p21 induction during C2 differentiation *in vitro* occurs significantly later than the block to the S phase, suggesting that p21 may play primarily a maintenance role in muscle differentiation (JW Harper and SJ Elledge, unpublished data). Clearly, much remains to be elucidated about the intimate connections between differentiation factors, CKIs and Cdks, but it is likely that the logic of the MyoD differentiation circuit will be repeated in other differentiation pathways.

A connection between development and cancer

Appropriate cell proliferation during development requires the expression of both cyclins to drive cell division, and negative regulators, such as CKIs and Rb, to block proliferation when required. It appears that these negative control pathways, present in several cell-type specific configurations, must be overcome during oncogenesis.

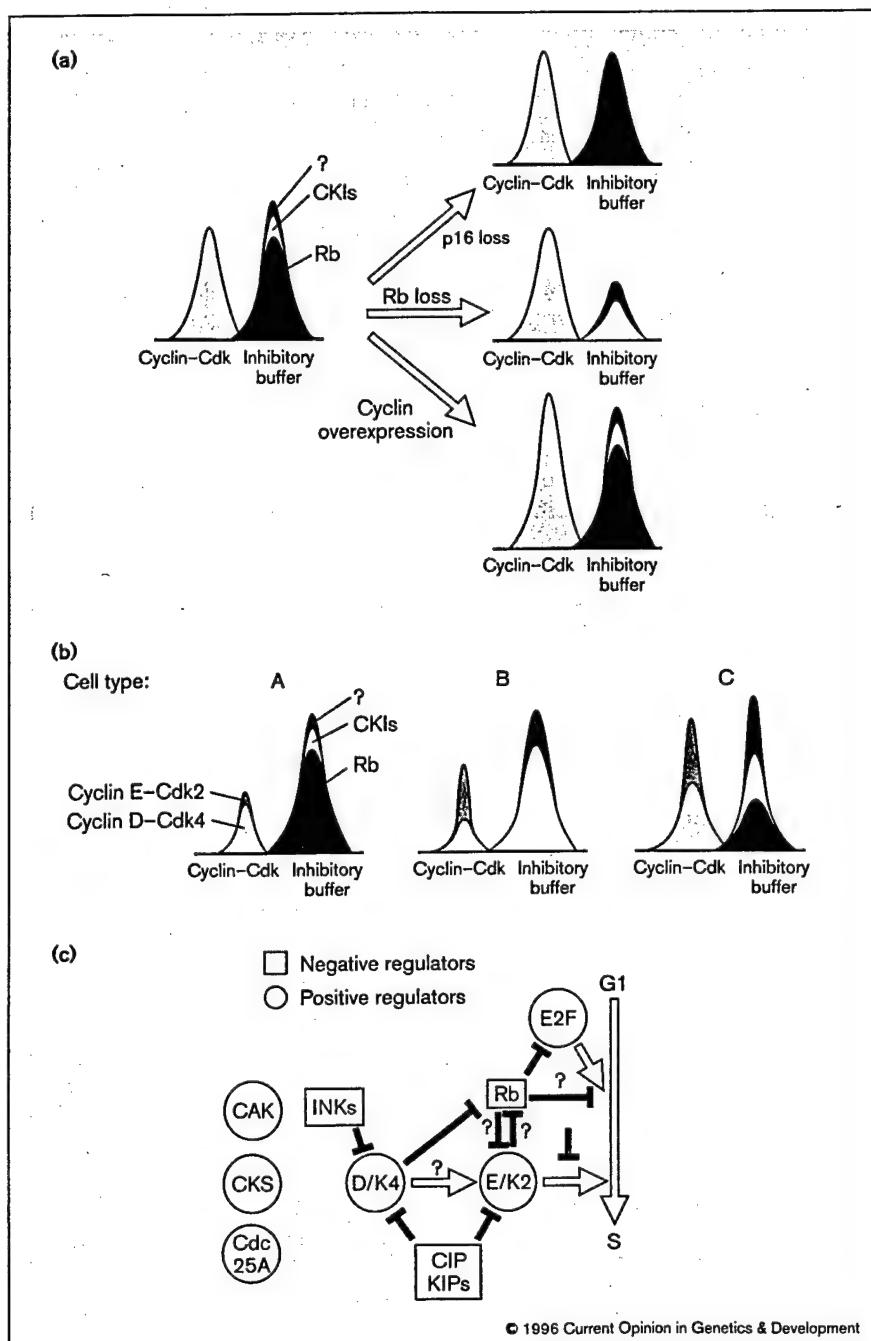
Figure 2



A model depicting a differentiation switch linking Cdks, differentiation factors and CKIs. Muscle-cell differentiation is set in motion through activation of the transcription factor MyoD, which functions as a heterodimer with E12. In the presence of appropriate growth factors, MyoD is kept inactive, possibly via phosphorylation. When certain growth factors are removed, a differentiation initiation signal is unleashed, leading to MyoD activation. MyoD activates transcription of a number of genes (including p21 and myogenin) that function to further the differentiation process. Ectopic expression of cyclin D1 has been shown to maintain MyoD in its inactive form even in the presence of initiation signals, suggesting that a cyclin-D-dependent pathway is normally responsible for maintaining MyoD in an inactive form in cycling myoblasts. Some of the genes induced by MyoD may inactive Cdks that normally keep MyoD inactive, thereby potentiating the MyoD-activation process. Other MyoD-dependent genes may function in a positive feedback loop to potentiate differentiation by maintaining MyoD in an active form.

Figure 3

Models for alterations in cell cycle regulatory machinery leading to loss of proliferative control. (a) Inactivation of negative growth pathways and cyclin overexpression. In arrested cells, cyclin-Cdk complexes required for entry to S phase are held in check by negative growth regulators including CKIs, negative phosphorylation (such as phosphorylation of Tyr15 in Cdk2), and Rb. These negative pathways serve as inhibitory buffers that block the ability of Cdks to phosphorylate critical substrates. Loss of a significant fraction of this buffer or re-activation of cyclin expression, such that the total cyclin-kinase activity exceeds that of the buffer, results in a kinase imbalance, leading to cell proliferation. Relative levels of components of the inhibitory buffer are indicated as black (Rb), white (CKIs), and light shading (other negative regulatory mechanisms such as tyrosine phosphorylation). The Y axis is a measure of the proliferative or inhibitory potential. (b) Levels of positive and negative factors present in terminally arrested cells display marked cell-type specificity. Relative levels of cyclin E/Cdk2 (dark shading) and cyclin D/Cdk4 (light shading) are indicated. (c) Scheme depicting the regulatory circuit for S-phase entry. Rb functions as a growth regulator in G₁, at least in part through its inhibition of E2F function. Phosphorylation of Rb by cyclin D-Cdk4 and perhaps cyclin E-Cdk2 is thought to inactivate its growth suppressive function. INK4 homologs selectively inhibit Cdks whereas CIP/KIP homologs inhibit multiple Cdks, including cyclin D-dependent and cyclin E-dependent Cdks. Rb may negatively regulate cyclin E and may also negatively regulate p16 expression in G₁. The regulators CAK, Cdc25, and CKS function at multiple steps and upon multiple Cdks during G₁ progression.



An example of the connection between cancer and development comes from the recent characterization of mice that lack cyclin D1 [56**]. This cyclin is expressed at unusually high levels in the retina and is required for retinal development. Presumably, the improper development of the retina in cyclin D1 mutants reflects an inability to overcome Rb, the proposed target of D-type cyclins. In what is clearly more than a coincidence, the retina is the same tissue in which high-frequency tumors arise in Rb-mutant humans. It is therefore likely that the Rb

protein is important in both development of that tissue and its maintenance in the non-proliferative state. A similar connection can be made for the cyclin D1-dependency of breast development during pregnancy [56**] and the occurrence of amplification of cyclin D genes found in breast tumors.

The emerging theme is that tissues that show a special dependency for particular genes to control their proliferation during development may be driven to malignancy by the

alteration of those same genes. Thus the developmental histories of tissues give us clues as to the nature of events required for conversion to the malignant state.

A central issue yet to be examined is the extent to which each of the known negative regulators contributes to maintenance of the non-proliferative state in individual cell types, particularly in situations where multiple mechanisms are at work. In principle, the control of cell proliferation can be viewed as a dynamic competition between positive and negative factors, each of which may be individually manipulated to control the cell cycle during development. In the dynamic competition model, negative regulators such as Rb and CKIs act as a potential energy barrier in the pathway that cyclin-Cdk must overcome to cause cell-cycle entry (Figs 3a and 3b). Removal of these barriers through mutation may reduce the levels of kinase activity required for cell-cycle entry, but some Cdk kinase activity is still necessary for the process of DNA replication and could therefore act as a target of further negative regulation (Fig. 3c). Cells reach the non-proliferative state via an increase in negative factors and/or a reduction in positive factors, such that the remaining positive factors (i.e. Cdk activity) are insufficient to overcome the negative potential. Non-proliferating cells may return to the proliferative state by enhancing the positive potential (e.g. increasing cyclin levels) or by decreasing the negative potential (e.g. reduction of CKI or Rb function) to a point where Cdk activity is sufficient to overcome this negative potential (Fig. 3a). In this scenario, different cell types will be capable of entering the malignant state by dismantling the particular constellation of inhibitors used by that cell (Fig. 3b); this would predicate the existence of tissue-specific tumor-suppressor genes.

The ability of these cells to proliferate once more may allow them to take advantage of additional alterations that occur during DNA replication as a result of mutations in tumor suppressors controlling genomic stability. Thus interplay between the different classes of tumor suppressors is likely. Class 1 includes the classical negative regulators of the cell cycle, Rb and the CKIs. Class 2 tumor suppressors act not by preventing growth directly, but by preventing mutations in class 1 genes and proto-oncogenes. Genes in this class include p53 and DNA-repair genes. As p53 can actively kill cells via activation of apoptosis, it may also be considered a growth suppressor. The third class of tumor suppressors may be the developmental regulators, and includes the WT1 gene, which is found as a mutant in Wilms' tumors, embryonic tumors of the kidney. WT1 is a transcription factor that controls kidney development. Mutations in this gene have been suggested to alter the developmental fate of kidney cells in such a way that they fail to properly terminally differentiate. If cells follow inappropriate developmental pathways, they may find themselves in a milieu where

the proper differentiation cues are absent, leading to inappropriate growth. It is possible that this class of tumor suppressors tie development to the proper control of class 1 tumor-suppressor gene expression.

As it stands, we have strong evidence for the involvement of the Rb and p16 genes in tumor suppression and there is some evidence for p21 being a tumor suppressor in the prostate [57]. It is possible that the Rb/p16 pathway will need to be inactivated in nearly every tumor type. Mutations in p21 and p27 in tumors are extremely rare [57] or absent [44,58,59], and it remains to be seen whether this is attributable to redundancy or whether they can act as suppressors in combination with other negative regulators. Other inhibitors such as p15, p16, p18 and p57 await investigation.

Conclusion

Our knowledge base of the links between development and cancer is in its infancy, and much remains to be learned. A clearer understanding of the connections between these processes will probably emerge as the phenotypes of mice lacking positive and negative cell-cycle genes are uncovered. More surprises are surely just around the corner.

Note added in proof

Previous studies examining 14 types of human tumors including 351 DNA samples have failed to identify loss of function mutations in the p21 gene. A recent study [57] has now identified mutations of p21 in a subset of prostate cancers. Of 18 patients examined, two had truncation mutations in the amino-terminus of p21 which are expected to be loss of function alleles. An additional mutation, Pro-4→Gln, was also found although it is not clear whether this is a loss of function allele. These results, coupled with the fact that p21 is normally expressed in the prostate epithelium [48°], suggest that loss of p21 may contribute to the development of some tumor types. In addition, the role of p21 in G₁ DNA damage function has been confirmed by Brugarolas *et al.* [63] using cells deficient for p21.

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TGF-β has long been known to arrest cells in the G₁ phase of the cell cycle. Previous studies suggested that p27 is involved in cell-cycle arrest by TGF-β as the former appears in cyclin E-Cdk2 complexes after TGF-β treatment. This paper reports the identification of p15 as a TGF-β-inducible inhibitor of Cdk4 and Cdk6. This result indicates that cell-cycle arrest is mediated by TGF-β via a complex mechanism and involves both transcriptional responses resulting in activation of cell-cycle inhibitors, and subunit interchange in which p21 family members are redistributed on Cdks (see [36]). Although p15 is induced by TGF-β, it has also been shown that p21 is TGF-β-inducible in some cell types (see [59]). Thus cell-cycle arrest by TGF-β may not be universally mediated via p15.

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An analysis of the expression of p21 during mouse development and in adult tissue. In general, the expression pattern correlates well with cells that have left the cell cycle, suggesting a role for p21 in cell-cycle arrest during development. In addition, experiments using p53-null mice indicate that expression

of p21 throughout development is not dependent upon p53. In addition, it is shown that p21 expression is induced by expression of the basic HLH transcription factor MyoD *in vitro* and that p21 expression correlates with myogenesis (also see [51**]).

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This paper demonstrates that expression of p21 *in vivo* is p53-independent but that induction of p21 after DNA damage is p53-dependent.

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This paper examines the expression of p21 in several human tissue using immunohistochemistry. In skin subjected to radiation induced DNA damage, p21 is expressed in a pattern consistent with its regulation by p53. Extensive analysis of p21 in the gastrointestinal tracts revealed a high degree of compartmentalization that correlated with proliferation rather than differentiation. This compartmentalization was essentially abolished in colonic neoplasm. These data indicate that mechanisms which disrupt the normal topological control of CKI expression may contribute to transformation.

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This paper reports that p21 expression is activated by expression of the basic helix-loop-helix (HLH) transcription factor MyoD *in vitro* and that induction of p21 correlates with conversion of myocytes to myotubes.

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See annotation [55**].

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Mice that lack cyclin D1 are viable but have defects in retinal development and proliferation of mammary epithelial cells in response to pregnancy. Cyclin D1 expression is highest in the retina. Together with the retinal phenotype, these data indicate that the development of this tissue is critically dependent upon cyclin D1. Similar requirements may exist in the breast epithelium. These relationships suggests that tissues that are driven to transformation by certain genes may also be dependent upon those genes for normal development.

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